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Abstract

The present review is aimed at elucidating relatively new aspects of mucoadhesion/mucus interaction and related phenomena that emerged from a Mucoadhesion workshop held in Munster on 2-3 September 2015 as a satellite event of the ICCC 13th –EUCHIS 12th. After a brief outline of the new issues, the focus is on mucus description, purification and mucus/mucin characterization, all steps that are pivotal to the understanding of mucus related phenomena and the choice of the correct mucosal model for *in vitro* and *ex-vivo* experiments, alternative bio/mucomimetic materials are also presented. Then a selection of preparative techniques and testing methods are described (at molecular as well as micro- and macroscale) that may support the pharmaceutical development of mucus-interactive-systems and assist formulators in the scale-up and industrialization steps. Recent applications of mucoadhesive systems (including medical devices) intended for different routes of administration (oral, gastro-intestinal, vaginal, and nasal) and for the treatment of difficult to treat pathologies or the alleviation of symptoms are described.

Contents

1. Introduction

The present review stems from a Mucoadhesion workshop held in Munster on 2-3 September 2015 as a satellite event of the ICCC 13th –EUCHIS 12th, held on 30 August- 2 September. We are perfectly aware that there are a significant number of reviews and papers already available in the current literature on mucoadhesion and on the relevant pharmaceutical applications. Thus, in order to avoid duplications, the present review represents an update of the topic but with special focus on some new aspects of mucoadhesion/mucus interactions, taking inspiration and advantage of the multidisciplinary nature of the above conference which gathered bio/medical as well as food technology and physical-chemistry experts.

2. Mucoadhesion

Definition of Mucoadhesion: It is common knowledge that mucoadhesion is a special case of bioadhesion, which is the ability of a material to adhere to a biological substrate. Thus in mucoadhesion the biological substrate is represented by mucosal tissue.

Opportunities and applications: The advantages are at least theoretically well understood. Mucoadhesive formulations are used to temporarily immobilize a delivery device on a specific site for targeted release and optimal drug delivery due to intimacy and duration of contact. Indeed it is no news that the short residence times of formulations (due to the various removal and dilution effects depending on the route of administration) at their intended site of action/absorption may result in reduced availability to the target tissue. Over the last 30 years, mucoadhesive polymers and formulations thereof have been developed for buccal, nasal, ocular, vaginal and oral applications. So far, a considerable number of papers focusing on the mucoadhesive properties of a wide range of polymeric materials have been published [1-3]. Such

a huge effort has not been paralleled by an increase of clinical applications which are still limited to a two digit number [4].

Mechanisms: Concerning the mechanisms, it is recognised and experimentally proven that the interaction between the mucus and mucoadhesive polymers is a result of physical entanglement and secondary bonding, mainly H-bonding and van der Waals attraction, which according the many authors, are mainly related to the following polymer properties: capability to create strong H-bonding, high molecular weight, sufficient chain flexibility, and surface energy properties favouring spreading onto mucus [5].

Testing methods: It has also to be recognized that a variety of *in vitro* tests have been developed by different research groups with the aim of understanding the phenomenon at different length scales, from observational (tensile testing, flow retention experiments) to molecular, using sophisticated techniques from fluorescence and confocal microscopy to a variety of spectroscopic techniques.

The new approaches-innovative aspects: In recent years, other concepts have emerged in the literature in relation to mucoadhesion. The first observation is that in many physiological situations the mucus layer is the main actor, and the focus should therefore be on its nature and complexity/variability depending on the anatomical site and on is sensitivity to various physio-pathological stimuli. It must also be recognized that mucins and mucus are quite different substrates and their interactions with mucoadhesives are different and should be taken into account when dealing with testing methods.

Mucus and food interactions: The comprehension of mucus interactions is also relevant in food technology for food progression and nutrient digestion and absorption. There is a need to link the knowledge acquired in this field to the problem of drug delivery.

Mucus penetration and mucoadhesion: Recently the focus has shifted to the mucus penetrating systems [6, 7] and the study thereof not as an alternative but as a complementary opportunity to mucoadhesion. They may work together to assure the best results.

Mucomimetic approaches: The recent trend aimed at the development of mucomimetics substrates to formulate in vitro mucus or mucosae model for both testing and innovative products should be recognised.

Summary: In line with the ongoing research in the field, the review will illustrate the latest preparative and testing techniques that may support the pharmaceutical development of optimized systems, intended for the different routes of administration. This knowledge is the driving force for the pharmaceutical and related companies in the field. In addition, the review aims to elucidate the above relatively new aspects of mucus characterization, mucus penetration and mucomimetic phenomena that represent the basis for a science-based development of any technological, *in vitro, ex–vivo* test and for any sustainable formulation development.

3. Mucus composition and properties as a function of location

Mucus is a highly complex viscoelastic medium that provides a defensive barrier for many different epithelial surfaces including the respiratory, reproductive and gastrointestinal (GI) tracts. It performs a range of functions including lubrication, maintenance of a hydrated layer and it acts as a barrier to pathogens and toxic substances while facilitating the exchange of gases and nutrients with the underlying epithelium.[8] The mucus layer comprises two different groups of mucins, secreted and membrane bound.[9] Membrane bound mucins form the glycocalyx that provides an important link between the cell surface and the secreted gel layer. On the luminal side of the membrane, these membrane bound mucins have either SEA (self-

cleaving) -domains (MUC1, MUC3, MUC12, MUC13 and MUC17) or von Willebrand domains (MUC4). The membrane bound mucins play a role in both cellular protection and signalling [10, 11] through mechanisms such as the regulation of chemokine secretion.

The secreted mucins are produced by submucosal glands and goblet cells and are characterized by their high molecular weight and high proportion of O-linked carbohydrate.[12] Mucus is continuously secreted with nearly 10L secreted into the adult GI tract alone.[8] The composition of mucus varies in different parts of the body. The mucins secreted into saliva are primarily MUC5B and MUC7 and comprise about 16% of the total protein in saliva [13], whereas the primary secreted mucin in the stomach is MUC5AC but with lower concentrations of MUC5B and MUC6. It is possible that small amounts of MUC5B found in the stomach are pulmonary in origin as pulmonary mucins are expelled via the GI tract. Intestinal secreted mucin is predominantly MUC2 but again there are low concentrations of MUC6 and MUC11 in the small intestine and MUC5B, MUC11 and MUC12 in the large intestine. Pulmonary secreted mucins are primarily MUC5AC and MUC5B [14, 15], both of which are considered to be gel forming. The secreted mucins of the female reproductive tract are primarily MUC5B but with lower concentrations of MUC5AC and MUC6.[16] In addition to the mucins the mucus layer contains lipids, salts, proteins, macromolecules and cellular debris.[17] In particular partially degraded cellular DNA provides a significant contribution to the viscosity of the mucus layer [18].

The properties of the various secreted mucins can vary significantly depending on the location but they are still largely controlled by the basic properties of the mucins. Thus, they are generally of high molecular weight (in excess of 1MDa) and are primarily hydrophilic. The extensive glycosylation means that mucins are stiff, extended polymers with a persistence length of 36 nm [19] and having a negative charge, often associated with sialic acid groups or sulphate. The properties of mucins in solution very much depends on concentration and what

other components are present in the local environment. The secreted mucins are normally considered to be gel forming.

In the GI tract the mucus layer varies widely in thickness. It is thickest in the colon and thinnest in the duodenum [20]. In the intestine the mucus barrier comprises two different regions, known as tightly adherent and loosely adherent.[21, 22] In the large intestine, these regions are clearly delineated and under healthy conditions the tightly adherent layer provides a physical barrier to bacteria. However, in the small intestine this layer is much thinner and the loosely adherent layer dominates.[23] Measurements of particulate diffusion through human cervical mucus has shown a network pore size of ~100 nm [24] and AFM images of intestinal mucin have shown a similar pore size. [25] Despite this data on intestinal mucus, as has already been stated, the small intestine is dominated by the loosely adherent layer, which is much more heterogeneous. This layer has been shown to allow the passage of even 2 μm particles provided that they carry a significant net negative charge. [18, 26] This will be discussed in more detail in Section 9.

4. Preparation of mucin or mucus

a. Purification of secreted mucins

There are very good books that describe the preparation of mucins, especially one edited by McGuckin and Thornton [27]. As a starting point, we recommend that secreted mucus is removed by gently scraping the epithelial surface with a plastic scraper and then purified [25]. Because of the large size and complex structure of secreted mucins it is important to use an extraction buffer containing a strong chaotrope capable of disrupting hydrogen bonding network. For example, 4M guanadinium hydrochloride has been widely used [28]. The resulting solution can then be purified using a two-step isopycnic density-gradient centrifugation, in which the first step removes proteins and the second step nucleic acid. Proceed by adjusting

the sample to a density of 1.4 g/mL with CsCl and centrifuge (55K rpm at 10 °C for 62 h). The high degree of glycation leaves the mucin strongly Alcian blue positive and this can be used to identify the mucin containing fractions. Aliquots of fractions can be sampled, absorption at 280 nm measured and 2 μL of each fraction can be spotted and stained with Alcian blue. UV and Alcian blue-positive aliquots should then be pooled and diluted in extraction buffer lacking guanidinium hydrochloride (final guanidinium concentration 0.5 M), adjusted in density to 1.4 g/mL with CsCl, and centrifuged again (50K rpm at 10 °C for 96 h). Again aliquots can be sampled, measured at 280 nm and stained with Alcian blue. The fraction at 1.4−1.55 g/mL and strongly Alcian blue-positive but with weak absorption at 280 nm is identified as the mucin fraction. More detailed methods for the purification of specific mucins can be gathered from the literature. For example, MUC5B [29] and MUC7 [30] from saliva, MUC5B from respiratory- and cervical-tract secretions[31] and MUC2 from intestinal mucus [25]. Confirmation of the presence of mucin resulting from the purification should be undertaken using immunoreactions. There is now wide range of antibodies available against mucins from a range of animal sources indeed for many mucins it is possible to target specific regions of the molecule.

Although the extraction and purification of mucins are well established methods, some disadvantages related to the short conservation time, lower yield of production and batch-to-batch variability lead frequently to the alternative use of commercial mucin. Commercial mucin the type from Sigma (Germany) or Orthana (Denmark) are purchased in lyophilized powder which then can be hydrated in ultrapure water or buffers for 3h at room temperature under gentle stirring. An extensive dialysis allows removals of small ions or low-molecular-weight additive. Several treatments have been reported in the literature. Rossi *et al*. increased the solubility of mucin from Sigma by adding 2% (w/w) SDS to 12% w/w mucins dispersion [32].

SDS was then removed by 2 days dialysis against 10 volumes of 1M urea-1M NaCl, followed by other 2 days dialysis against 40 volumes water and finally against 0.1 M acetate buffer pH 4.5. Alternatively, the mucin dispersion can be centrifuged for 1 h at 25,000xg, the supernatant fraction collected, lyophilized and stored at 4°C until usage. The glycoprotein concentration can be measured by colorimetric method or absorbance reading at 280 nm and calculated on the basis of the difference before and after the treatment. Samples from Orthana have been characterized in terms of monosaccharides composition revealing a predominant presence of neutral O-linked oligosaccharides which confer high hydrophilicity and high solubility up to 200 mg/mL. Solution of this commercial mucin can be prepared by dispersion in water, extensive dialysis and finally lyophilized. Orthana as well Sigma mucins do not show the gelling properties upon lowering pH however rheological studies demonstrated the existence of a concentration-dependent variation of the viscosity from dilute to semi diluted to entangled state [33].

In addition to preparative methods such as those mentioned above, analytical methods such as agarose gel electrophoresis can also be used and separation monitored by lectin, immunochemical or histochemical staining. This method can be used to analyse minimally treated samples as long as they are protected from degradation. As the separation is based on the inherent charge of the mucin, it can be used to separate different mucins [34] or different glycated forms of the same mucin. [35]

b. Biomimetic approaches

In parallel with biological mucin and mucus, efforts to develop artificial mucus or mucus models have been put forth for long. Easily accessible mucus models or mimics are beneficial for all research disciplines requiring mucin, mucus or mucosa for a number of obvious reasons;

biological mucus could be difficult to be accessed by some research groups, generally cumbersome to prepare, and presents ethical issue.[36] Technically, biological mucus samples may reveal inconsistent structure and properties across studies due to differences between individual animal sources and/or preparation details. While some mucus models have been devised clearly in the context of mucoadhesion and drug delivery, some others have been developed for other purposes and thus may be considered for future mucoadhesion studies. Growing interests in mucus models are reflected in a few excellent review papers on this subject published in recent a couple of years, including by Groo and Lagrace[37] and Authimoolam and Dziubla[38] with a focus on artificial mucus, and by Cook and Khutoryanskiy[36] with a focus on artificial mucosa, respectively. Briefly, mucus models can be classified into glycan micro-arrays,[39-42] mucin layers,[43-47] complexes of mucins with synthetic polymers,[48-52] and synthetic polymers,[53-56] roughly according to the scale. Glycan micro-arrays have gained popularity for its specificity in probing glycan-binding receptors, antibodies, and enzymes,[39, 40] and can be applicable to mucoadhesion too. Despite that micro-arrays with mucin-specific glycan arrays are also readily available,[41, 42] application in the context of mucoadhesion is rare probably due to the lack of three dimensional, mechanical barrier character in those systems. In fact, this is a common problem for all other types of two dimensional mucus models, such as various monolayers of mucins on substrates.[43-47] Mucin-synthetic polymers complexes were motivated from that self-aggregated mucins, especially commercially available ones, in aqueous solvent even at physiological concentration or higher do not reproduce viscoelasticity comparable to that of native mucus.[57] Thus, synthetic polymers, especially mucoadhesive polymers, are employed as crosslinker to enhance the network forming capabilities of mucin aggregates. Representative polymers include guar gum/borate,[50] alginate,[48] poly(acrylic acid),[49] and glutaraldehyde.[52] Some hydrophilic and network-forming synthetic polymers, such as locust bean gum/tetraborate,[53] poly(acrylic

acid)/(hydroxypropyl)methyl cellulose,[56] poly(styrene) sulfonate,[55] and poly(ethylene glycol)-*block*-poly(lactic acid),[54] have been employed even without involving mucin molecules. The assessment of synthetic polymeric systems or complexes of mucin and synthetic polymers as mucus models has typically been conducted via characterization of rheological properties[48-53] and adhesive properties (detachment forces) against mucoadhesive drug tablets,[56] often in comparison with biological mucus. These two properties represent mechanical integrity of mucus model and their interfacial chemical properties against mucoadhesive polymers, respectively, in the context of drug delivery researches. Nevertheless, no mucus model or mimic that can universally replace biological mucus has emerged yet, presumably because of diverse and complex properties required for mucoadhesion researches.

5. Preparation of electrospun mucoadhesive formulations

During the last two decades, electrospinning has gained increasing interest as a promising technique for biomedical applications.[58-60] In drug delivery, nanofibers are appealing due to their high encapsulation efficiency and flexible encapsulation capacity.[61] Moreover, electrospun fibers allow for numerous delivery and encapsulation options; blend, core-shell, particles combined with fibers, etc[62, 63]. Electrospun fibers have a large surface area that allows for extensive interactions with the surrounding environment, which, depending on the application, can be mucus or other biological components. Surprisingly, mucoadhesion of nanofibers has not yet been extensively addressed. From the limited studies (examples from the literature can be found in Table 5.1), it is evident that the mucoadhesive properties of nanofibers can be manipulated by changing nanofiber properties, such as the extent of cross-linking.[64-66] Moreover, the inherent mucoadhesive properties of some biopolymers can be exploited when developing mucoadhesive nanofibers. Thus, biopolymers with known adhesive properties (such as alginate and chitosan) have been electrospun with increased bioadhesion of

the nanofibers compared to those made from synthetic polymers.[67, 68] However, the physico-chemical properties of nanofibers does not necessarily correlate with those of the unprocessed material,[69] for which reason mucoadhesion of biopolymeric nanofibers in general must be studied. Also, the effect of fiber morphology on the mucoadhesive properties, such as fiber diameter, is yet to be explored.

The oral mucosa is permeable and vascularized, and therefore an appealing delivery.[70] The group of Yang developed a delivery system for the oral mucosa, based on a semi-interpenetrating network (sIPN) made from gelatin.[71, 72] By cross-linking the fibers using polyethylene glycol diacrylate (PEG-DA) the authors obtained stable, mucoadhesive fibers. The mucoadhesion was affected by several factors: stability, porosity, swelling, and PEG composition of the scaffold.[71] The sIPNs were used as a delivery system for insulin, and the authors found that the transbuccal permeability of the released insulin was larger than that of free insulin.[71] Another delivery system targeting the oral cavity was developed by Tonglairoum *et al.*, who developed polyvinylpyrrolidone/cyclodextrin/clotrimazole sandwich patches coated with chitosan (CS) or thiolated chitosan (CS-SH) for oral candidiasis.[73] The authors studied the fiber's mucoadhesion, and thus the ability to adhere to the oral mucus. It was shown that fibers coated with CS-SH exhibited a higher mucoadhesive strength compared to CS coated, which is in line with thiolated chitosan providing stronger interaction with the mucus.[73] The mucoadhesive properties of nanofibers can also be controlled by adding mucoadhesive small molecules. For instance, Wongsasulak *et al.* obtained increased mucoadhesion of zein–chitosan composite electrospun fibers by addition of alpha-tocopherol (a-TOC).[65, 74] Electrospun nanofibers have also been studied for vaginal drug delivery.[75, 76] In a study by Zong *et al.*, polyethylene oxide (PEO)/polylactide composite electrospun nanofibers was developed and loaded with cisplatin for local chemotherapy. The mucoadhesive

properties of the nanofibers caused the fibers to stay in the vagina and release the drug, whereas the gel leaked out. Accordingly, the nanofibers facilitated increased bioavailability of the drug as compared to a gel.[75] Electrospun fibers have shown promising results for mucosal drug delivery, however the full potential is still to be revealed.

Table 5.1 Examples of electrospun formulations for drug delivery.

Mucosal target	**Fiber material**	**Drug**	**Ref**
Buccal mucosa	chitosan or thiolated chitosan/polyvinyl alcohol	Garcinia mangostana extract	[77]
Buccal mucosa	polyvinyl alcohol	Di- phenhydramine	[66]
Buccal mucosa	polyvinyl alcohol	Docetaxel	[78]
Buccal mucosa	Gelatin and photo-reactive polyethylene glycol diacrylate	Nystatin, insulin	[71, 72]
Buccal mucosa	chitosan/polyvinyl alcohol	Clotrimazole	[79]
Buccal mucosa	polyvinylpyrrolidone/cyclodextrin/clotrimazole and chitosan/polyvinyl alcohol	Clotrimazole	[73]
Sublingual mucosa	polyvinyl alcohol and sodium alginate/polyvinyl alcohol	Insulin	[64]
GI mucosa	polycaprolactone	Diclofenec sodium	[80]
Gastric mucosa	Zein, chitosan and poly(ethylene oxide)	α-tocopherol	[65, 74]
Vaginal mucosa	polystyrene coated with poly(allylamine hydrochloride) or dextran sulfate sodium	HIV entrapment	[81]
Vaginal mucosa	cellulose acetate phthalate	TMC 125/Viread	[76]
Vaginal mucosa	poly(ethylene oxide)/polylactide	Cisplatin	[75]

Ocular mucosa	Polyvinyl alcohol/polycaprolactone	Timolol maleate and dorzolamide hydrochloride	[82]

6. Methods for molecular scale testing of mucoadhesion

a. Spectroscopic studies

Over the last 20 years a range of spectroscopic methods have been used for the in vitro analysis of the mucoadhesive behaviour of polymeric materials, and the determination of their affinity toward mucin at the molecular level.[83-85] In particular, the interactions between glycoproteins\mucins with mucoadhesive polymers have been investigated by *^{1}H and/or ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy* or by *NMR diffusion* measurements. Analysis using NMR is advantageous, as no sample derivatization or pre-treatment is needed and due to the advantage of non-alteration of the normal bio-functionality of the biomolecules. Uccello-Barretta and co-workers have used proton selective relaxation rate NMR measurements for the determination of mucoadhesive properties of different polysaccharides.[86] Mucoadhesivity can be determined by exploiting the possibility to detect changes of affinity to mucin of small probe molecules due to the mucin–polysaccharide interactions. They have demonstrated the affinity of ketotifen fumarate (KT) to mucin, and they have used KT as an interaction probe to compare the bovine submaxillary mucin affinities of tamarind-seed polysaccharide and larch arabinogalactan.[87] Diclofenac sodium salt has also high affinity for mucin (and low affinity for the polysaccharides), and was also employed as mucoadhesivity probe for polysaccharide mixtures containing tamarind seed polysaccharide and hyaluronic acid.[88] It has been shown that the selective relaxation rate of the ligand is a

more sensitive indicator of binding than the non-selective relaxation rate is. Earlier studies using ^{1}H and ^{13}C Nuclear Magnetic Resonance, indorsed that the hydrogen bonds formed between the carboxylic acid of poly(acrylic acid) and the glycoprotein component of mucus, play a significant role in the process of mucoadhesion.[89, 90] Moreover, Griffiths *et al.*, has used pulsed-gradient spin-echo (PGSE-NMR) diffusion measurements to study the interactions of various model polymer therapeutics with mucin and to quantify their diffusion within mucin solutions.[91] A strong interaction with mucin was observed for a series of polyamidoamine dendrimers and hyperbranched poly(ethylene imine), which displayed a characteristic pH-dependent profile and led to significant reductions in their rates of diffusion.

The use of *attenuated total reflectance-fourier transform infra-red* spectral analysis *(ATR-FTIR)* is another spectroscopy method to study the interfacial interaction/absorption, and the diffuse phase across the interface of mucoadhesive polymers and mucins segments.[92] Sriamornsak *et al.,* studied the mechanisms of gastrointestinal mucoadhesion of different pectin films in contact with mucin in different media.[93] The diffusion of water was used as an indirect measurement of any change resulting from the interpenetration of polymer–mucin chains at the aqueous solution-polymer film interface.[94] The ATR-FTIR data confirmed the formation of hydrogen bonds and the changes resulting from the interpenetration of pectin–mucin chains at the film interface. Furthermore, by using ATR–FTIR spectroscopy Xiang and Li suggested that intra-polymer interactions, and inter-surface interactions played opposite roles in the mucoadhesion performance of cationic polymers, at the negatively charged buccal mucosa surface.[95] The intra-polymer interactions can increase the crosslinking within the polymer and lead to the decrease of mucoadhesion, while the inter-surface interactions can promote mucoadhesion of the polymer. Optimal mucoadhesion can be achieved by balancing these two interactions. In a recent study, ATR-FTIR was used to investigate the molecular

interactions between a chitosan hydrogel (consisting of non-ionic surfactant vesicles, niosomes, with chlorotoxin) and various cell lines for cancer therapy. The specific accumulation of mucoadhesive chitosan on the surface of ovarian epithelial carcinoma cells was confirmed, demonstrating chitosan's specificity in targeting of mucin antigen overexpressing tumor cells.[96]

Several of the mucoadhesive studies focus on bulk polymers, however, interest in the mucoadhesion at the nanoscale has been growing the last years.[97, 98] In fact, the mucoadhesion ability of nanoparticulate systems to mucin is affected by their surface properties (hydrophobic, hydrophilic), surface charges and their size. To detect the mucoadhesive phenomena in the intestinal tract after oral administration of nanoparticulate systems, *confocal laser scanning microscopy* (CLSM) has been used.[99, 100] Chen *et al.*, investigated the adhesion of chitosan-modified liposomes, (average diameter of ~200 nm) using CLSM and fluorophotometry with coumarin 6 as the fluorescent probe.[101] Their studies indicated that the positively charged surface charge of the liposome particles played an important role in their interaction with the negatively charged mucin fibres. In another study, the *in vivo* mucoadhesion of pH-responsive thiolated chitosan nanoparticles for oral low-molecular weight heparin delivery was assessed using CLSM.[102] Fluorescein-5-isothiocyanate (FITC)-labelled nanoparticles were prepared and the intensity of green fluorescence in the small intestine epithelium of rats after oral administration were evaluated. It is to note, that the CLSM method is sensitive to detect the organic dye-labelled association of nanoparticles to the mucosal layer of the animal intestine, and does not modify the properties of the developed formulations of the nanoparticles. Instead of organic fluorescence materials, orally administered quantum dots (QDs, semiconductor nanocrystals with diameters of 1–10 nm), could be used as fluorescence markers. Tahara and co-workers have developed QD-loaded liposomes which had high biocompatibility and low

toxicity in Caco-2 cells.[103] By using CLSM, the fluorescent signal of QDs in the liposomes could be detected in the intestinal mucosa after oral administration. Thus, QDs can be used for tracing and detecting bioadhesion and uptake of liposomes in *in vivo* applications. The relaxation NMR approach, using dexamethasone 21-phosphate as a mucoadhesivity probe, confirmed the *in vitro* mucoadhesivity of nanoparticles obtained from quaternary ammonium chitosan conjugates.[104] The high surface area of nanoparticulate aggregates is remarkably enhancing the interactions with bovine submaxillary mucin. In addition to nanoparticles and liposomes, block polymeric micelles were also tested for the development of mucoadhesive drug loaded nanovehicles. The mucoadhesivity of solutions of micelles having acrylated end groups was characterized by using ^{1}H NMR.[105] To quantify the extent of reaction, the decreased area under the curve in the vinyl proton regime of the NMR spectra, (indicating interactions between the acrylates and thiols present in cysteine residues of the mucin), was evaluated.

Overall, spectroscopic studies are very useful to investigate the interactions between polymers or nanoparticulate systems with mucus. The choice of the mucoadhesion spectroscopy method affects the characterization of their bioadhesive\diffusion properties and the determination of the mucoadhesive strength.

b. Atomic Force Microscopy

Atomic force microscopy (AFM) is another method that has been used in mucoadhesion measurements. The imaging mode can provide essential information about the amount and conformation of material adhering to the sample, while force spectroscopy enables sensitive adhesion measurements. In order to increase the surface contact area between the tip and the sample in force measurements, it is advantageous to prepare a so-called ‘colloidal probe’. As

shown in Figure 6.1, a colloidal-sized particle is attached to the AFM cantilever using two component epoxy glue. The colloidal probe and the sample surface can be further functionalized with molecules of interest (mucin, APTES, -COOH, -NH_3, -OH groups, antibodies and others). Later on, the cantilever is moved towards the surface in the vertical direction. The deflection of the cantilever is measured during the approach and retracts of the probe; as a result, a force-distance profile is obtained. The maximum force of adhesion (F_{adh}) and the work of adhesion (W_{adh}) can be determined from the retract curve.

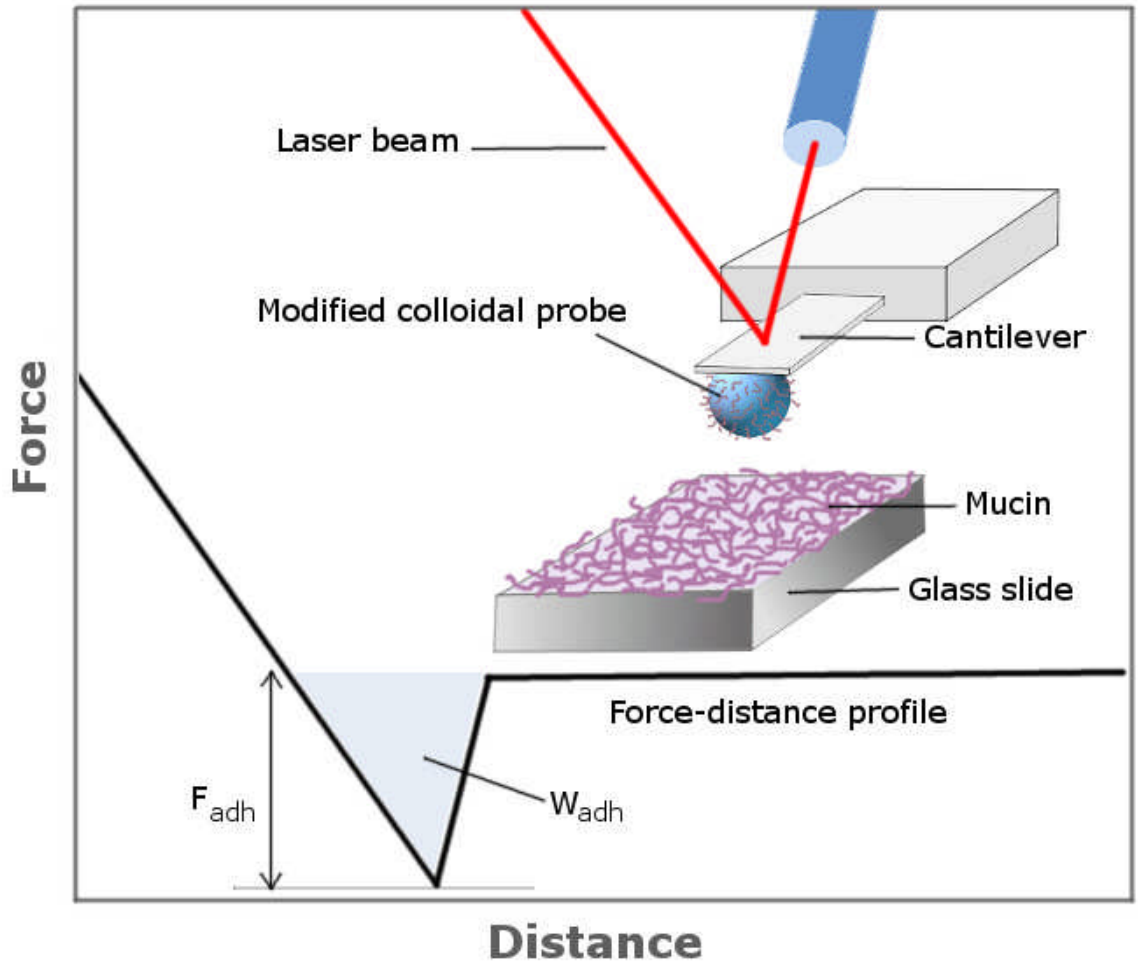

Figure 6.1 Scheme of experimental setup and force-distance profile for mucoadhesion measurements.

The colloidal probe approach has been used by Cleary *et al*. in order to measure the adhesion between a Pluronic-PAA modified glass bead and the mucous substrate [43]. The mucoadhesion

was studied in conditions of varying pH and ionic strength. It was also found that the time of contact between the probe and the sample affects the adhesive forces. Prolonged contact favors interdiffusion and interpenetration of polymer chains and mucin network, resulting in increased adhesive force. Pettersson and Dedinaite investigated the interactions between mica surface and silica particles coated with mucin and mucin-chitosan layers [106]. In order to mimic the daily oral care procedure and its influence on mucous layers, the films were exposed to the anionic surfactant SDS. Another interesting approach to the colloidal probe method was presented by Iijima *et al*., who have measured the interactions between mucin layers and stimuli-responsive drug delivery vehicles [45]. Instead of using the colloidal sized, glass or silica particle attached to the AFM cantilever, the nanogel particles were freeze-dried and the resulting granules were directly adhered to the tip by means of micromanipulation system.

Joergensen *et al*. used the image analysis of AFM scans in order to evaluate the mucoadhesive properties of different pectins [107]. Mucin coated mica was scanned in AFM liquid cell before and after incubation with polymer solution, followed by comparison of the roughness parameters extracted from the images. Sriamornsak *et al*. investigated the structures of mucin, pectin and their mixtures in acidic medium and deionized water, observing formation of large aggregates in neutral pH conditions [47]. Similar study by Deacon *et al*. assessed the interactions between pig gastric mucin and chitosan [108].

AFM in mucoadhesion measurements presents both advantages and limitations. It allows sensitive force measurements as a function of pH, ionic strength or time of contact, but it is also time-consuming and can be affected by a choice of place in the case of heterogeneous samples.

c. Scattering techniques (SAXS, SANS, SLS and DLS)

The detailed macromolecular structure of mucin has been addressed at molecular level using high-resolution scattering techniques, namely, synchrotron SAXS [109-111], SANS [110, 112] and static and dynamic light scattering [33]. This has allowed accounting for the properties of mucin samples of different biological origin and methods of preparation. Thus, the cylindrical model, and more recently, the double-globular (or "dumbbell") comb model, has been used to describe the complex mucin structure [33, 109, 112]. The schematic structure of mucin at different length scales and its mechanical response at varying pH are represented in Figure 6.2.

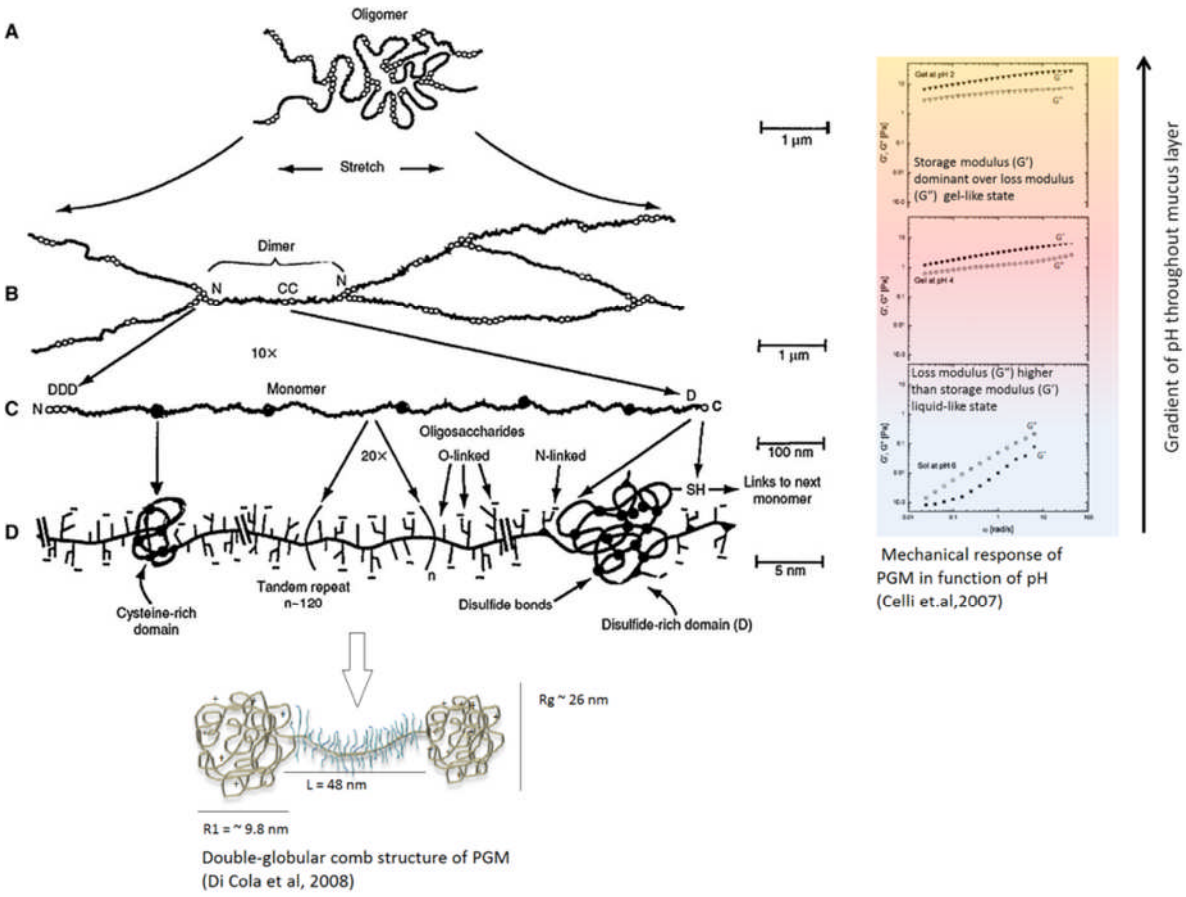

Figure 6.2. Schematic representation of the biochemical structure of gel-forming mucin at different magnifications: A) entangled mucin network; B) mucin monomers cross-linked through disulfide bonds ; C) mucin monomer with globular naked-protein regions and D) low scale representation of the bottle-brush highly glycosylated region of mucin (Sources: Modified

from [8, 113]; mechanical spectra of pig's gastric mucin (PGM) as a function of pH taken from Celli *et al*. [114]; double-globular comb structural parameters taken from Di Cola *et al*. [109] and corresponding to pharmaceutical mucin sample "Orthana" in aqueous medium in absence of salt). With permission of American Chemical Society and Elsevier.

Table S1 summarizes the results of biophysical studies, based on scattering techniques, namely, synchrotron SAXS, SANS, and SLS and DLS, that have addressed the structural properties of purified mucins of different biological origin. Fundamental parameters probed include the radius of gyration (R_g), the coil overlap concentration (c*) and the slope of the intensity scattering curves (also known as the fractal dimension (d_f)). These parameters have been determined at varying conditions of pH, solvent, concentration and temperature. The wealth of documented studies has contributed to the elucidation of the mechanisms and molecular events that govern the properties of mucin which underlie its biological functions such as the formation of gel networks. Indeed, mucin participates in the formation of the gel which prevents the digestion of stomach epithelia caused by the acidic gastric juice. This is a function of pH, but also mucin concentration and ionic strength. At physiological conditions, the high concentration of mucin (> 20 mg/mL) and the high-molecular-weight of the molecules, favor the formation and stabilization of an entangled network which behaves as a weak reversible gel [115]. On the other hand, mucin undergoes to sol-gel transition [114, 116] a low pH (pH < 4) due to a conformational change in which hydrophobic domains of the non-glycosylated cysteine-rich regions become exposed and the negative charges of the sugars residues responsible of maintaining the expanded structure get protonated. As observed *in vitro* for native mucin, this phenomenon is accompanied by increase of the size at pH ~2 [117, 118] due to aggregation of mucin by a combination of hydrophobic and electrostatic interaction and

entanglement of the sugar chains resulting in an increase of the viscosity of the solutions [114, 119]. In support of the model proposed for mucin gelation, AFM images have shown that mucin is in an extended fiber-like shape at pH 6.0, whereas it forms well-defined clusters at pH 2.0 [117]. Consequently, the different conformation of mucin throughout the mucus layer allows selective diffusion of HCl. At low concentration [116], in presence of high ionic strength [119], commercial mucin [57], does not gel. However, pH-dependent interactions, as shown by DLS and CD-spectroscopy, are attributed to a conformational transition of mucin at pH < 4.0 [120, 121] that imparts some fluidic viscoelasticity to the bulk sample [114].

Recent studies using synchrotron SAXS have aimed to gain insight into the interaction between soluble commercial pig gastric mucin and alginates of high-molecular-weight (~ 400 kDa) and low-molecular-weight (~4 kDa) [122]. Firstly, the structure of mucin alone (at 3 mg/mL), at three different values of pH, namely at 1.2, 2.5 and 4.0, was investigated. The scattering curves were characterized by a single fractal dimension, df = -1.6 at pH 4.0, which at low-q range, increased to df = -2.6 when lower pH were assessed. This observation is consistent with a pH-driven conformational transition in the mucin, in agreement with observations in other mucin samples differing in origin and preparation methods, as revealed from other techniques. The structure of mucin in three different concentration (namely, at 0.3, 1.5 and 3.0 mg/mL) was characterized by different scattering profile, being the one at lower concentration ideal to calculate the radius of gyration (R_g) that afforded a value of ~18 nm. Interestingly, when the more diluted mucin was mixed with two different types of alginates, different effects in the high-q range of the intensity scattering plot were observed. Indeed, the addition of the low-molecular-weight alginate produced a scattering profile in which the high-q range resembled the one of mucin at high concentration (3 mg/mL). By contrast, this effect was less pronounced when adding the high-molecular-weight alginate, where the high-q region resembled more closely the behavior of mucin at low concentration. Based on this evidence, along with that from fluorescence

quenching spectroscopy, viscosimetry and DLS studies, a general model was proposed to explain the interaction of soluble mucin with polyanions. This model accounts for the influence of molecular weight, charge and degree of chain contraction (Figure 6.3). Although the overall net charge of mucin is negative, positively charged patches are expected to occur in the non-glycosylated protein globular regions of mucin due to the presence of histidine, arginine and lysine. These positive patches represent sites for the interaction with negatively charged polysaccharides.

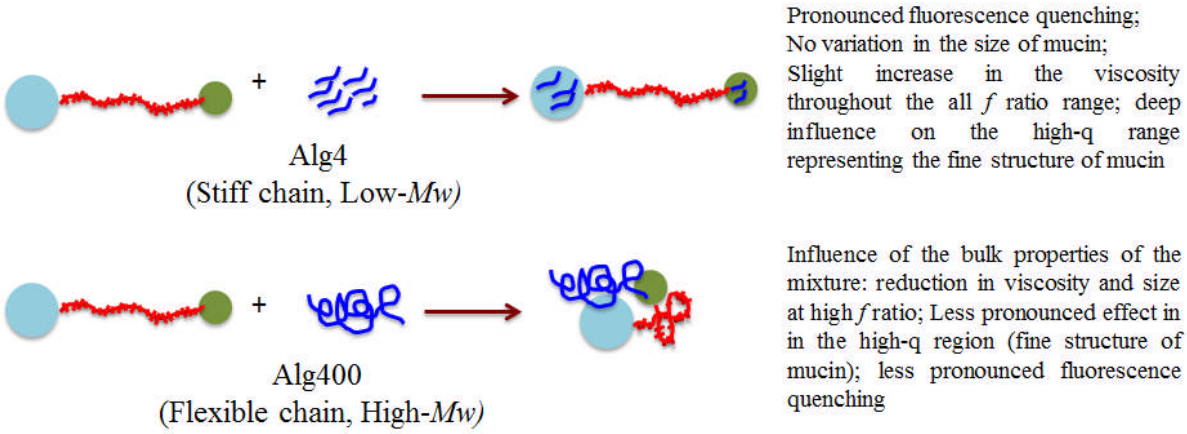

Figure 6.3 Model of interaction between the mucin in its double-globular comb mucin structure and alginate as a function of alginate´s Mw (Alg 4 = 4 kDa; and Alg400 = 400 kDa) and chain flexibility [122]. With permission of American Chemical Society.

Low-molecular-weight and stiff polyanions will interact mainly with the sites available on the globular regions without influencing the preferred conformation of mucin. Thus, minimal variation of the bulk properties such as size and viscosity are expected to occur. However, due to the small size, low-molecular-weight polyanions are able to penetrate in the globular structure inducing eventually rearrangement of the protein. On the other hand, high-molecular-weight and more flexible polyanions, due to the large size, might act as bridges between distant

available sites thus influencing the initial conformation of mucin and favoring a reduction of the overall hydrodynamic volume.

7. Methods for macroscale testing of mucoadhesion

The methods to study mucoadhesion can be classified depending on the underlying physical phenomena involved and also depending on the type of formulation that can be tested. Table 7.1 and Table S2 summarize the investigative techniques available. In this Section, we focus on those that probe macroscale phenomena.

Table 7.1. *In vitro* methods used to study mucoadhesion as classified on the basis of the physical phenomena involved.

Test method	Formulation	Mucosal surface/mucosa mimetic material/mucin
Methods based on the mechanical force determination [123]		
Texture Analyzer	Compressed polymers tablets [56]; Polymers solutions [124]; Casted polymer films [125, 126]; Polymer gels [127-129]; Compacted polymer microparticles into tablet [130]	Animal mucosal tissue [126, 128, 130]; Mucosa-mimetic hydrogels [56]; Mucin-coated (Sigma) filter papers [124, 125]; Mucin (Sigma) disc [127]; PGM (Sigma) gels [129]
Modified balance/modified surface tensiometer	Polymer coated glass [131]; Compressed polymer [132, 133]; Polymer cups [134]	Animal mucosal tissue [132-135];

Tensile tester	Polymer paste [136]; Hydrogels [137]	Plexiglas® disk [136]; gelled BSM [137]
Tensile stress tester	Composite hydrocolloids [138]	Filter paper [138]
Rotational cylinder	Compressed polymer tablets [1, 133]	Animal mucosal tissue [1, 133]
Atomic Force Microscopy (AFM)	Polymer coated glass microsphere [43]; Polymer solution [139]; Mucin-polymer complexes [47, 108]	Human buccal cells [139]; Freshly purified PGM [108]; PGM (Sigma) [47]
Methods based on mucoadhesive interaction		
Surface Plasmon Resonance (BIACORE®)	Covalently-bound polymer on CM5 chip [140]	Submicron-sized commercial PGM suspension [140]
Dynamic light scattering (DLS)	Mucin-polymer complexes [121, 141]	
Turbidity	Mucin-polymer complexes [121, 142]	
IR-NMR	Freeze-dried mucin-polymer mixed solutions [143]	Crude homogenized porcine gastric mucus [143]; PGM solution [143]:
Analytical ultracentrifuge	Polymer-mucin mixed solutions [144-146]	PGM from different gastric regions [144]; HGM [145]

Impedance crystal quartz microbalance (QCM)	Polymer solutions; Polymer-micelles [147]	BSM (Sigma) solution [147]
Method based on flow forces		
Flow through systems	Fluorescent labeled nanoparticles [148, 149]; Polymer microparticles [150]	Ocular tissue [148]; PGM (Sigma) solution [149]; Isolated small rat intestine [150];
Method based on fluorescent probes		
Fluorescence determination	Fluorescent labeled-nanoparticles [151]; Fluorescent labeled-polymer solutions [152, 153]; Polymer solutions [154]	Animal mucosal tissue [151-153] Pyrene-labeled human conjunctival epithelial cells [154]
Multiple Particle Tracking	Fluorescent particles [155]	Purified PGM hydrogels [155]
Method based on rheological solution properties		
Viscometer	Polymer-mucin mixed solution [122, 142, 156, 157]	Mucin (Sigma) solutions [122, 124, 142, 157-159]; Homogenised porcine gastric mucus [160, 161]
Rheometer	Polymer-mucin mixed solution [124, 141, 142, 158, 159]	

a. Rheology including polymer interaction in dilute solution

The interaction occurring between mucus and mucoadhesive polymers in mixed systems produces variation in the flow properties of the mixtures respect the ones of the single components. Thus, the study of the rheological properties of mixtures of mucus or mucin in solution with mucoadhesive polymers has been widely exploited. Steady-shear measurements of viscosity, η (defined as the resistance of a fluid to the imposed shearing force), and oscillatory shear determinations of the mechanical viscoelastic moduli (namely, storage and loss moduli, G' and G'', respectively), have been used to study liquid and gel-like systems, respectively [162]. In general, when two different macromolecular species (*e.g.*, polysaccharide and protein) are mixed in solution, either attractive or repulsive interactions can take place [163] (Figure 7.1). Attractive interactions can result in the formation of a complex that either remains as a soluble colloidal complex or else precipitates as a coacervate. Repulsive interactions in turn, depending on the concentration of the macromolecular species, can lead to phase separation or co-solubility [163]. In the case of associative interactions, the bulk viscosity of dilute mixed solutions is expected to decrease due to overall reduction of the hydrodynamic volume of the macromolecules when they are combined. However, in some other cases, cooperative intra and inter-polymer interaction can induce increase in viscosity which is higher than the expected sum of the individual contribution, up to physical gelation. This "synergistic" interaction was previously observed in xanthan and galactomannan or in plasma proteins and egg albumin mixed system [162, 164]. In repulsive interactions, the viscosity of mixed solutions is expected to remain similar to those of the individual stocks. However, if the conformation of one of the molecules changes due to the exclusion into a segregated phase, then the viscosity of the mixture can also deviate from the expected additive line. Viscosity synergism cannot distinguish between binding interaction and exclusion effects [165], unless experimental criteria are applied. In the experimental conditions in which polysaccharides and mucin solutions are

in the dilute regime (η_{rel} ~2; η_{sp} ~ 1), polymer exclusion effects are assumed to be negligible [162] being the polymers well below the overlap coil concentration.

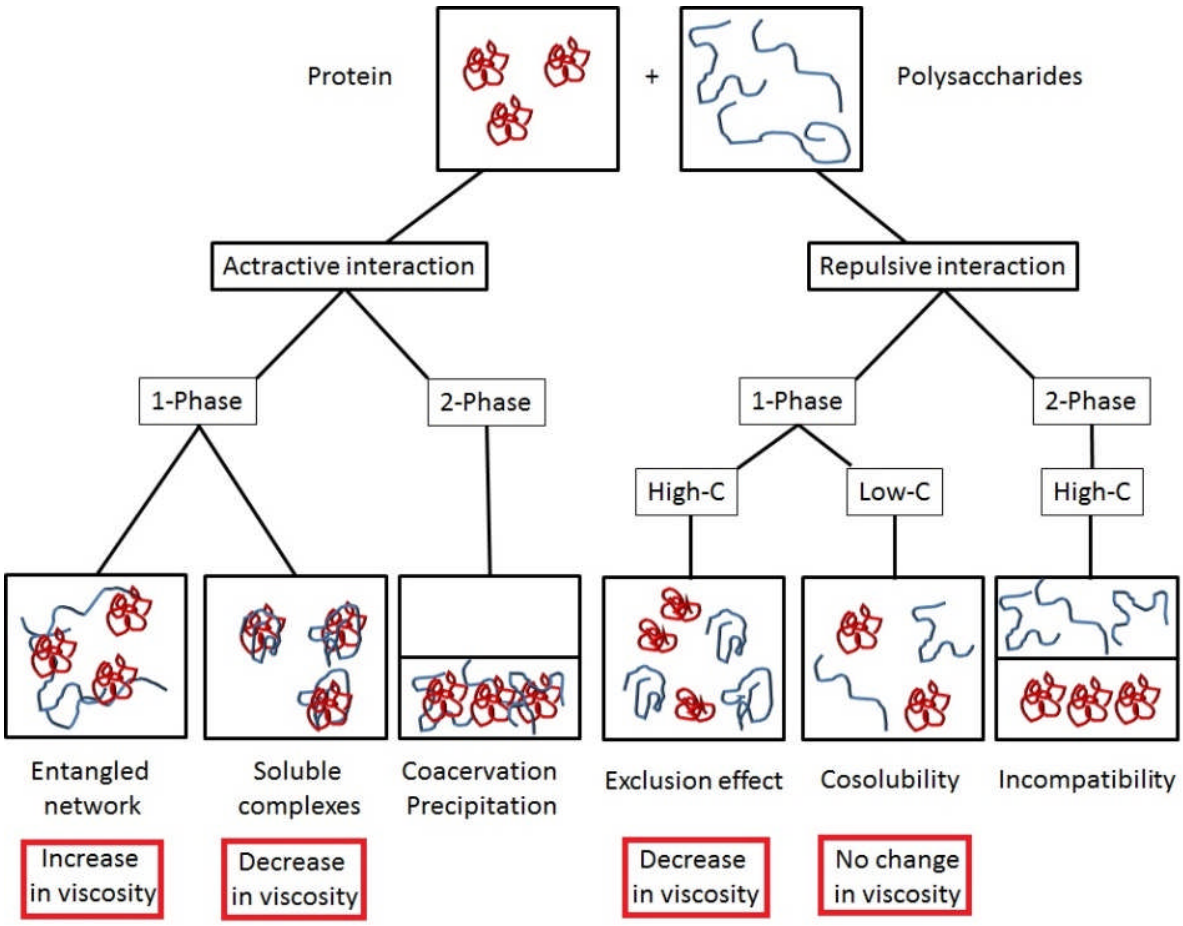

Figure 7.1 Schematic representation of the type of interaction that can occur in protein-polysaccharides blends in dilute solution mixtures (modified from [163]).

Mucus is a weak viscoelastic gel biological material which possesses both flow (viscosity) and deformation (elasticity) properties [113]. Such properties are regulated for example during peristaltic movement or copulation [8]. At higher concentration mucus is characterized by a shear thinning behavior (*i.e.* decrease in viscosity upon increase of the shear rate) typical of an entangled network. However, the soluble fraction of PGM (Sigma) at concentration of ~ 8 mg/mL (in 0.1 M TRIS pH 7.4) was found to behave as Newtonian fluid since any shear-

dependence of the viscosity was observed [157]. The addition of human albumin produces an increase in viscosity due to association of albumin and mucin [157]. In the context of mucin and polymer interactions, a rheological approach to screen the mucoadhesive properties of polymer was described by Hassan and Gallo [156]. The mucoadhesion strength of several polysaccharides was evaluated by studying the viscosity enhancement occurring upon mixing solution of polymers with commercial mucin sample using a viscosimeter Brookfield Model RTV (Brookfield Engineering Laboratories, Stoughton, MD). The increase in viscosity (positive synergism) respect to the sum of the individual viscosities of the two components measured in the same conditions as the mixture (in terms of concentration, temperature, time and rate of shear) but with an Ostwald capillary viscosimeter (Fisher Scientific Co., Pittsburgh, PA) was attributed to physical entanglement between the two species and defined as component of bioadhesion (η_b). For each polymer-mucin system, η_b was calculated with the following equation:

$$\eta_{b\,t} = \eta - \eta_m - \eta_p \qquad \text{Eq. 1}$$

where η_τ is the measured viscosity of the mixture and η_p and η_m the individual viscosity of the polymer and mucin, respectively.

The η_b values were found to be inverse proportional to the rate of shear per second (σ), thus, the force of bioadhesion F, defined as intermolecular friction force per area unit was calculated using the equation:

$$F = \eta_b \times \dot{\gamma} \qquad \text{Eq. 2}$$

Based on this pioneering protocol, several subsequent studies aimed to test the mucoadhesion of polymers. This procedure could distinguish between positive synergism (interaction), lack of synergism (no-interaction) or negative synergism of the viscosity or of the mechanical properties. Mortazavi *et al*., [166] reported the gel strengthening effect of poly-acrylic acid on

homogenized mucus and observed that was characterized by increased values of G' (which reflects the ability of a viscoelastic material to store the elastic energy and recover its initial shape) and decrease in G'' (which reflects the loss of energy as liquid-like flow). Madsen *et al*., [160] described the effect of mucoadhesive type and concentration on the profiles of the mechanical spectra of the mixtures in order to determine the type of gel formed. Some of the most relevant works based on rheological methods that have contributed to a systematic description of the mucoadhesive properties of polysaccharides are summarized in Tables S2 and S3. Sometimes, different outcomes have been observed for the same polymer-mucin mixture, such as in the case of chitosan-mucin [142, 156] or cellulose derivative-mucin [159, 160] depending on different experimental conditions, particularly the polymer concentration [158] or mucin source, making direct comparisons and interpretations challenging [167].

Recent evidence [168], has shown that mixing two stock solutions of chitosan and mucin of matched $\eta_{rel} \sim 2.0$, at increasing *f* ratio (mass proportion of mucin respect the total mass in the mixture) a reduction in η_{rel} to a minimum value ($f_{\eta min}$) occurs beyond which, upon a subsequent increase in *f*, the η_{rel} increases again to approach that of mucin stock solution. Such behavior describes a skewed U-shaped curve both in water and 0.1M NaCl (pH 4.5) as shown in Figure 7.2a and b, respectively, for a representative CS-mucin systems. This approach enables to determine, in a quantitative manner, the degree of interaction, given by the value of the area under the curve of the relative deviation from the theoretical additive line (or line of "no interaction"). Also, the method enables to determine the maximum stoichiometry of the interaction given by the *f* ratio of minimum η_{rel} ($f_{\eta min}$).

Mechanical force studies or rheological synergism are diagnostic of mucus (or mucin)-polymer interactions, however, no detailed information regarding the underlying molecular mechanisms of interaction can be deduced from these techniques. Table S3 offers a summary of the major

rheological methods that have been used to study the interactions of polymers and proteins with mucin solutions and mucus gels.

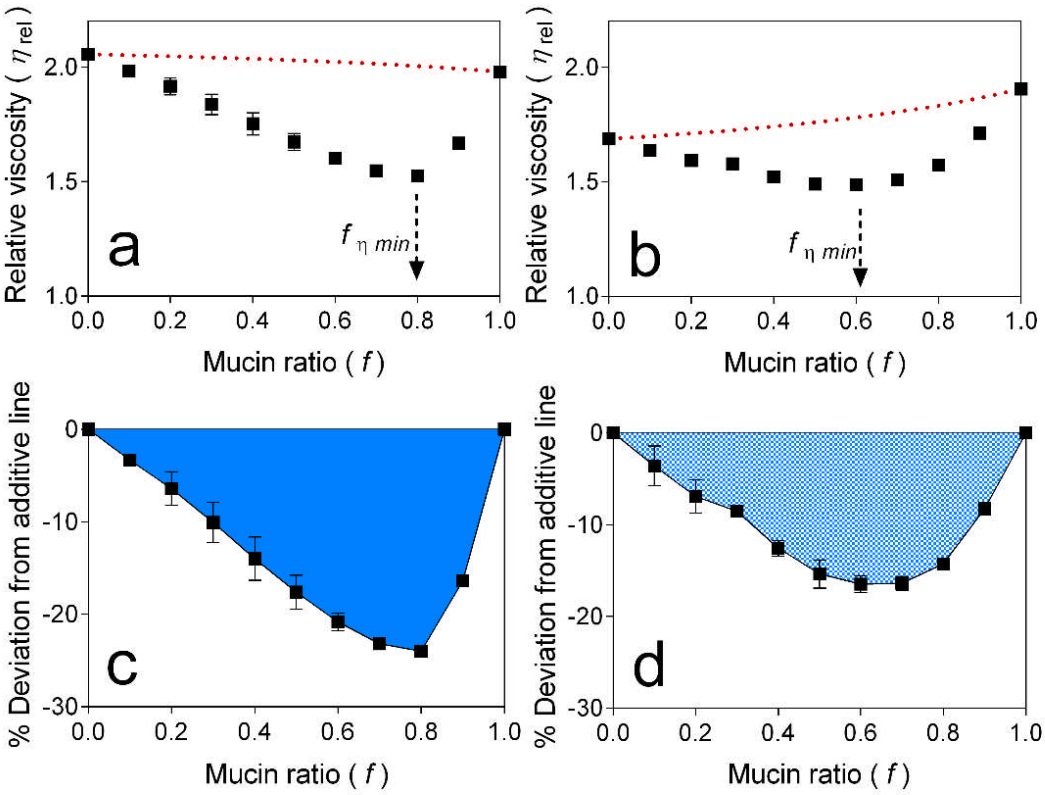

Figure 7.2 Relative viscosity (η_{rel}) of chitosan–mucin mixtures of varying compositions expressed as the mass fraction of mucin (f) respect the total mass in *a)*water and *b)*0.1M NaCl (37°C, pH 4.5, inclination angle 50°). The red dotted line in a) and b) represents the calculated values of η_{rel} of the mixtures assuming there is no interaction (additive line). The η_{rel} values at f = 0 and 1 are the relative viscosities of the chitosan and mucin stock solutions, respectively. The lower panels show the normalized data expressed as percentage deviation from the additive line in *c)* water and *d)* 0.1 M NaCl, both at pH 4.5 (mean values ± minimum and maximum, n=2). The blue shaded areas in plots *c)* and *d)* represent the integrated area under the curve calculated using a trapezoid approximation available in Origin 8.5 (Origin Lab Corp., Northampton, MA) [168].

c. Inclined plane

As pointed out in the introduction, a variety of methods can be used to study mucoadhesion and in Table 7.1 a classification of methods is given based on the physical phenomena involved. From a practical point of view it is useful to distinguish between mechanistic methods and functionality (or performance) test methods; the first ones (the most common are rheological and spectroscopic methods) give information on the events that occur at the mucoadhesive joint in order to prove the interaction mechanisms, whereas the second ones are aimed at evaluating the actual mucoadhesive properties/performance of formulations. In turn, they can be divided into mechanical tests (the most common are tensile testing and rotational cylinder) intended to measure the force needed to detach the formulation from the substrate and dynamic tests (among which flow through or flow retention methods) intended to mimic the physiological clearance mechanisms and to follow the fate of the formulation/loaded drug (retention on or removal from the mucosal substrate). Mechanical and dynamic methods are believed to provide information on the overall performance of the formulation as a delivery system.

The inclined plane method [169, 170] can be classified as a special dynamic test that measures mucoadhesiveness as a function of the retention of the mucoadhesive material in contact with a mucosal substrate (mucin film or mucosal tissue). It has been devised to test liquid or semisolid formulations endowed with intrinsic flowing properties at test temperature. It is not applicable to solid formulations or very thick gels.

Description of the apparatus

The inclined plane apparatus basically consists of a plexiglas support whose angle of inclination with respect to the horizontal can be varied between 30° and 60°, thermostated at 37°C and placed above an electronic microbalance interfaced with a personal computer. An

illustrated picture of the apparatus, including details of the plexiglass support (which is composed of a thermostated plate and an adapted substrate holder) is given in Figure 7.3.

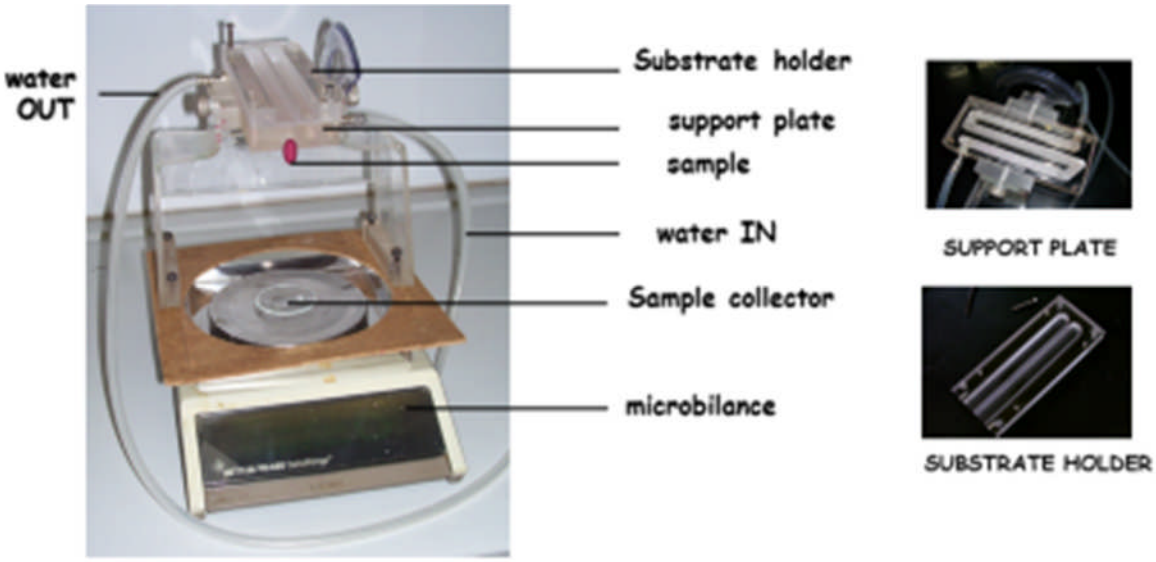

Figure 7.3 Illustration of the inclined plane apparatus

The substrate holder (hosting two parallel channels) may be coated with a thin mucin film (prepared by casting) or covered with mucosal tissue. The surface area coated is normally 28 cm^2. The whole apparatus is placed in a transparent box allowing constant temperature to be maintained and avoiding disturbances during the measurements. An overall picture of the assembled apparatus is given in Figure 7.4.

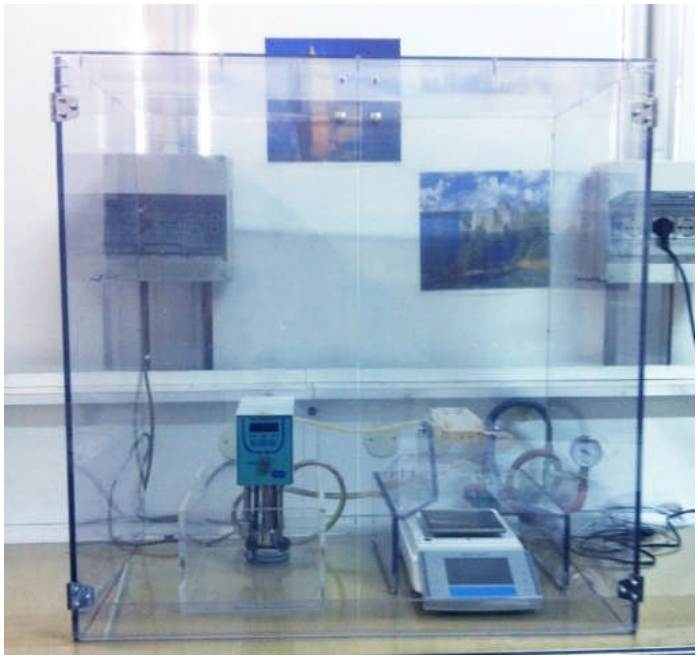

Figure 7.4 Overall picture of the assembled apparatus

Description of the operational procedure for measuring mucoadhesive properties

The substrate holder is coated with the mucin film and equilibrated. Porcine gastric mucin is normally used as biological substrate. Mucin films are prepared directly on the Plexiglas holder in the horizontal position, by pouring a measured volume of 8% w/w mucin dispersion in water then drying at 45°C for 45 min. A weighed amount of the formulation is placed on top of the substrate holder, still held horizontal and until equilibrated. The support plate is then inclined (at a given angle) and the amount of formulation dropped on the microbalance is recorded as a function of time. Blank measurements are performed in the absence of the mucin film on a weighed amount of sample using the same experimental conditions employed in the presence of mucin. The amount of formulation dropped down the inclined plate is recorded by means of suitable software as a function of time until a plateau is reached. The amount adhering to the inclined plate is calculated as the difference between the amount of formulation loaded and the amount dropped down from the balance (non-adherent) and expressed as a percentage (% adhered). An example is given in Figure 7.5.

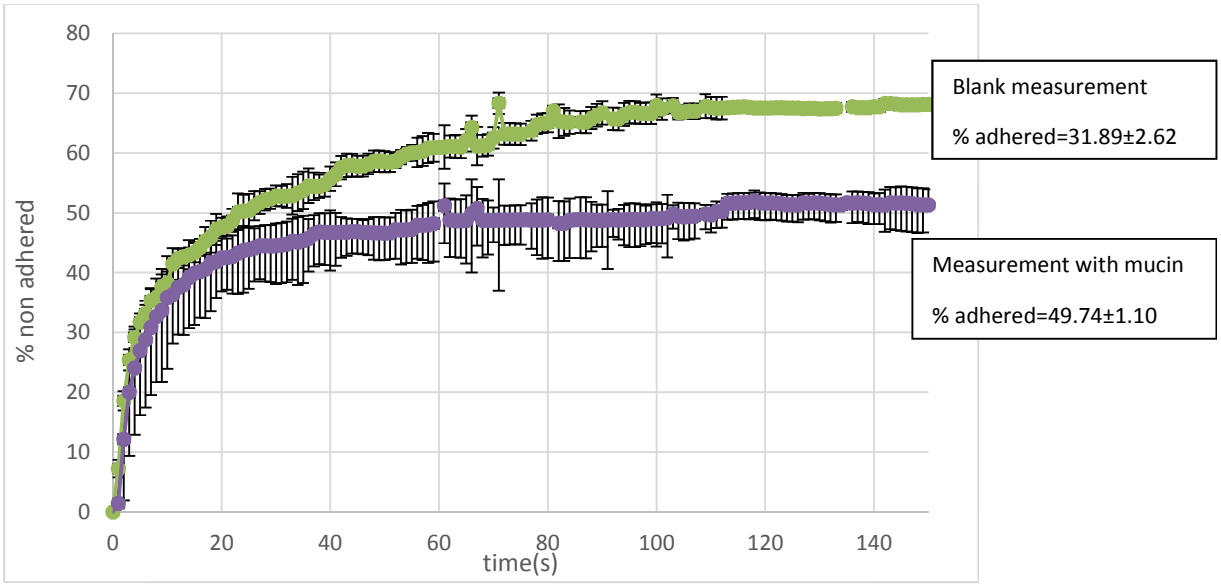

Figure 7.5 Plots of the amount of sample dropped (non- adherent) on the balance as a function of time.

A normalized mucoadhesion parameter is calculated as follows: (% adhered mucin-% adhered blank)/% adhered blank and is equal to 56%.This parameter allows the mucoadhesive properties of a given formulation to be measured independently of the consistency of the sample, since the blank measurement allows for normalization [170]..

Validation of the method

The inclination angle, quantity of mucin, length and width of the channels engraved on the sample holder, sample weight influence test results and their reliability and must be optimized to manage sample and testing variabilities. Recently these parameters have been the object of a validation exercise aimed at 1) evaluating the capability of the method to discriminate between different prototypes of a formulation intended for marketing and 2) assessing the precision and reproducibility of the method as well as the robustness with respect to operational parameters. This exercise could lead to the proposal of the method as a routine control method for the quality of the product.

Applications

The method has been profitably used to test the mucoadhesive properties of polymeric solutions, liquid or gel formulations (mouthwashes, vaginal washings, eye drops, buccal sprays, nasal washings, nasal sprays) [170] and even melted suppositories. The method has also been employed to test mucoadhesive systems characterized by in situ gelling properties, like swallowable gels intended for esophageal lining, or in situ gelling solutions used in diagnostic colonoscopy, since it enables to evaluate the contribution of gelation time to mucoadhesive performance [171].

d. Tensile testing

A texture analyzer can be used for the quantification of the tensile strength *i.e.* the force required to remove the formulation from a mucosal surface, which can be used as a measure of the mucoadhesive strength. In a generalized setup, the formulation is fixed on a probe which is subsequently lowered into a mucus sample. After incubation that ensures full contact between the formulation and the mucus, the mucoadhesive strength is measured as the force of detachment. Formulations such as tablets [171], films, [172, 173] hydrogels, [174] and fibers [73, 82] can be studied using this technique.

8. Cellular methods

Use of mucosal tissue of animal origin in mucoadhesion studies presents certain drawbacks, such as limited availability, time-consuming tissue preparation, small surface area, or significant variability of the obtained results [175]. *In vitro* study of mucoadhesion on cell cultures is an example of alternative method of measurements, based on the interactions between mucin and material of interest. Mucus-secreting HT29-MTX cell line is derived from human colon adenocarcinoma and often used as a model for human intestinal adsorption or buccal tissue in the oral cavity. Mucus secretion is depending on the culture period and usually

reaches maximum thickness after ca. 3 weeks of cell growth [176, 177]. Figure 8 presents the HT29-MTX cells 1 and 14 days after passaging, respectively.

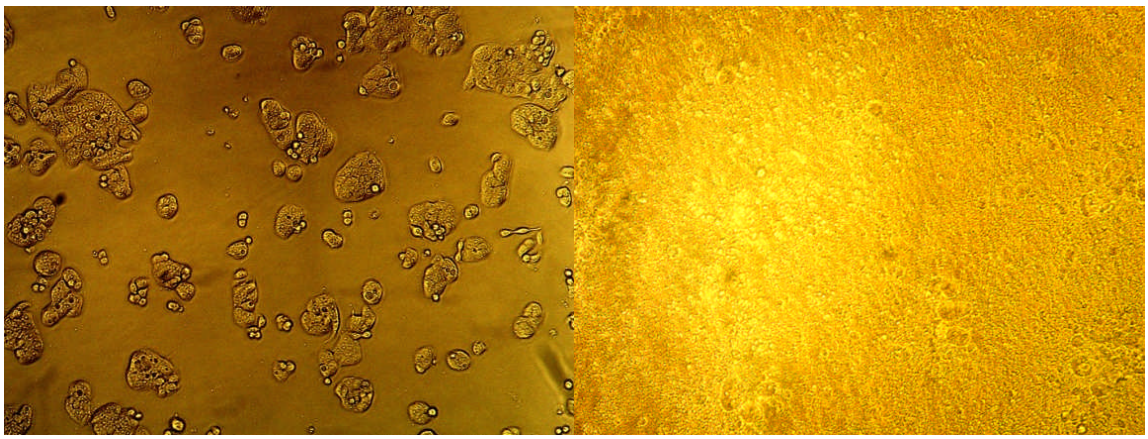

Figure 8.1 Morphology of the HT29-MTX cells 1 (a) and 14 days (b) after passaging.

There are several publications reporting use of cellular methods in mucoadhesion measurements. Jintapattanakit *et al.* assessed the mucoadhesion of trimethyl chitosan and PEGylated chitosan using HT29-MTX-E12 monolayers [178]. Fluorescently labeled polymers were incubated with cells for 2 hours. Later on the cells were lysed and the uptake of polymers was determined as the amount of fluorescence per unit weight of cellular protein. The obtained results were similar when compared to mucin particle method. Chen *et al.* evaluated the mucoadhesion of probiotic alginate microcapsules by counting *Lactobacillus reuteri* strain released from the capsules adhering to the HT29-MTX after 1 hour of incubation. [179] In another study, mucoadhesion of fluorescently labeled, non-coated and polymer-coated liposomes was measured after 2 hour of incubation with HT29-MTX cell monolayer [180]. The amount of liposomes adhering to the mucus was determined by measuring the fluorescence intensity both directly on the cell monolayer and indirectly from the supernatant solution after incubation.

9. Methods for characterising mucus permeability

One of the primary functional roles of mucins is to provide a barrier to bacteria while at the same time allowing the passage of smaller components such as nutrients or gasses depending on location. As a result the permeability of mucus and factors that can affect it are of great interest. The permeability of mucus depends on the pore size of the network and the size of the objects trying to pass through it. There have been essentially two types of approach used to assess permeability, either measuring the diffusion of particles through mucus from different sources or measurement of the pores directly, or indeed inferring it from the mucin structure. There have been a number of studies undertaken using the first approach and involving the tracking of particles of different sizes to determine pore size [24, 181-183]. The advantage of using particles of a well-defined size is that the diffusion coefficient can be used to calculate the microviscosity of the mucus sample (Stokes viscosity). For example, a study on human cervicovaginal mucus using multiple particle tracking, revealed that the average pore size 340 +/- 70 nm and that the range was approximately 50–1800 nm [183]. For smaller particles, fluorescence recovery after photobleaching (FRAP) has been used to determine diffusion coefficients in mucus [24]. Using this method it was proposed that human cervical mucus had a pore size of 100nm. This approach has also been extended to include gastric and intestinal mucus, MUC5AC and MUC2 respectively. In this case the diffusion of 500 nm latex beads was determined as a function of mucin concentration. The results showed that lowering pH caused both mucins to gel and the addition of a polyphenol (epigallocatechin gallate) to porcine gastric mucin caused a similar effect. A combination of FRAP and particle tracking has been used to show that soluble dietary fiber can decrease the permeability of porcine intestinal mucus [184]. Another approach that has been used is the use of optical tweezers to probe the microrheology and particularly, the rigidity of the mucus scaffold in a noninvasive way on the micrometer scale. [185] These approaches are in marked contrast to others who have tried to

estimate intestinal mucus permeability based on structural parameters [186]. This would indicate a pore size of about 1 micron for intestinal MUC2 mucus. Such a size is significantly larger than that measured by AFM in reconstituted MUC2 mucin that suggested a distribution of pore sizes from 20 to 200 nm [25].

In addition to the pore size, the charge on mucus is a key factor in determining permeability. The net negative charge carried by the mucins means that any positively charged particles or polymers tend to be mucoadhesive. This has led to the widespread use of cationic biopolymers such as chitosan for their mucoadhesive properties. In the small intestine, it has been shown that 500 nm latex beads can diffuse through intestinal mucus when bile salts adsorbed to the surface while they were unable to do so in the absence of bile. [26] In the same article the authors were able to show that *E. coli* were unable to diffuse through the same mucus regardless of the presence of bile. This suggested that a zeta potential of at least -20 mV was required for permeability of these particles.

10. Application specific requirement

a. Gastrointestinal drug delivery

Oral delivery is the most commonly used and compliant drug administration way. MMoreover, the all gastrointestinal tract (GI) is characterized by a highly absorptive surface which plays a role both for local and systemic effects. An important disadvantage of this administration route is however represented by the harsh conditions of the stomach which pose challenging goals for the development of carriers for the delivery of poorly stable drugs such as antibiotic and proteins and which goes towards the production of always more innovative and complex micro- and nanoformulations. In this context, the mucus layer underlying the all GI tract, with its variable in thickness and composition, represents the major protective barrier against foreign

particulate (e.g., bacteria but also micro-and nanoformulations). Nevertheless, this location represents also an important anchoring site for mucoadhesive drugs formulations which avoids their rapid clearance and improves their residence time in all the GI.

Several diseases affect all GI including accessories organs such as liver, pancreas and bladder. Gastrointestinal diseases cover acute, recurrent and chronic diseases including inflammatory bowel disease (IBD) and functional dyspepsia. Diseases of the upper part of the gastrointestinal tract are often associated with the hostile presence of *Helicobacter pylori*, the spiral-shaped Gram-negative bacteria which infect about half of the world population and establish life-lasting bacteria-host relationship [187]. Because of the lack of symptoms in ~ 60% of the infected people, its presence as a commensal or pathogenic bacteria is still controversial [188]. However, in a relevant percentage of the cases, the perpetuation of the inflammation of the gastric mucosa produces tissue damage that turns into more severe pathologies such as gastric and duodenal ulcer, adenocarcinoma of the distal stomach or MALT-lymphoma [189].

b. Advances in the therapy of *Helicobacter pylori*

The first-line therapy for the management of *H.pylori* infection, based on the concomitant assumption of a proton-pump-inhibitor and a combination of two antibiotics (i.e. triple therapy), is facing failure in ~ 20-30 % of the cases. This daunting efficacy is due to the alarming increase of the antibiotic resistance in association with low patients compliance and different disease's status, high bacterial load and polymorphism between strains and poor drug stability in the acidic environment [190, 191]. Improving drug stability, gastroretention at pH 1.2 and release at *H. pylori* surviving condition (pH 6-7) and site-specific targeting on *H. pylori* surface are therefore the major outlook for new tailored and effective eradication therapy. Along with the preparation of dosage forms whose prolonged residence time in the stomach is due to their ability to float in the gastric fluid as the consequence of their low density[192], or

to unfold and expand as a result of their swelling properties [193, 194], great attempts have focused on the formulations of polymeric micro-or nanoparticles with enhanced mucoadhesion ability [195]. Several research groups have demonstrated proof-of-principle of the superior antimicrobial activity of mucoadhesive micro- or nanoformulations both *in vitro* and *in vivo* with respect to the plain drug [196-198].

Besides the large portfolio of synthetic polymers such as poly-acrylic acid (PAA) and poly(lactic-co-glycol acid) (PLGA) and derivatives with proven mucoadhesive properties, proteins, and polysaccharides, used singularly or together like building blocks of drug delivery systems, also represent optimal materials. Among their advantages, include their biodegradability, biosafety, ubiquity, nutritional value but also amenability to being chemically manipulated [199]. In this regard, nanoparticles comprising gelatin [200] or gliadin (gluten-derived proteins) have been developed for the release of amoxicillin [198], clarithromycin-omeprazole [201] or clarithromycin-amoxicillin-omeprazole [202] for the treatment of *H. pylori*. While alginate, heparin or chitosan are the first choices for the formulation of mucoadhesive polysaccharide-based micro-and nanoformulations. The following section aims to review some strategies adopted to improve the performance of mucoadhesive polymer-based micro-and nanoformulations for gastric drug delivery.

Micro- and nanoformulation with improved gastroretention

Among mucoadhesive polysaccharides, chitosan, the semi-synthetic cationic aminopolysaccharide derived by partial deacetylation of chitin, remains the most adopted mucoadhesive biopolymer for the preparation of matrix-type or core-shell micro- and nanosystems for the delivery of low-molecular-weight drugs such as antibiotics. But chitosan is also a unique building block of galenic formulations due to its adjuvant properties and antimicrobial activity toward pathogenic bacteria like *H. pylori* [203, 204]. Chitosan-based micro-

or nanoparticles can be prepared by ionotropic gelation with tripolyphosphate (TPP)[205], covalent crosslinking and emulsification techniques [206]. The high solubility of chitosan at low pH [207] and the high porosity of chitosan-microspheres, however, restrict its applications in controlled release devices in the gastric compartment. Manipulating the crosslinking properties [208] or the solubility of chitosan [209] represents, therefore, a strategy to overcome this limitation. Exposing chitosan microsphere to reacetylation with acetic anhydride can modulate the release of amoxicillin or metronidazole with respect the un-reacetylated formulation but can decrease the encapsulation efficiency of metronidazole and also the antimicrobial activity if the exposure time is too high [209]. Also, chemical crosslinking of chitosan microsphere with glutaraldehyde [210] or with genipin [211], the low cytotoxic agent derived from hydrolysis of geniposide, can prevent their rapid dissolution in simulated gastric fluid. Nevertheless, this procedure can adversely influence the mucoadhesive properties of the microsphere if the crosslinking is superior to optimal time [211]. Zhu *et al*. [212] solved the inconsistency between mucoadhesives and controlled release by encapsulating a model drug into Eudragit® cores into chitosan/gelatin microsphere only slightly crosslinked with TPP. In this study, the authors evaluated the effect of type and density of crosslinking regard swelling properties, mucin adsorption on the surface and *in situ* retention [212]. To protect chitosan-glutamic acid nanoparticles from rapid dissolution at low gastric pH, Chang *et al*. [213] proposed an original approach. They included the nanoparticles in a pH-sensitive gel comprising alginate-Ca^{2+}-gelatin which would adhere first to the gastric mucosa, would shrink at pH 1.2 and protect the nanoparticle from rapid dissolution, swell up to 50% at pH 4.5, and then collapse at pH 7 allowing 80% release amoxicillin-nanoparticles. Beside chitosan, improved mucoadhesive properties can be achieved using other polymer mixtures such as dextran derivatives (*e.g.*, dextran sulfate) and cellulose acetate [214], cholestyramine and cellulose acetate butyrate [215] or ethylcellulose and carbopol-934P [216].

pH sensitive formulations

Due to its almost unique property among the biopolymers of bearing positive charges along its chain, chitosan is often used in combination with other mucoadhesive negatively charged polymers such as alginate (Arora *et al* 2011) or heparin [217, 218] to form polyelectrolyte complexes. Because of the formation of polyelectrolyte complexes is often enthalpically driven, (*i.e.*, they are formed mainly by electrostatic interactions or hydrogen bonding), they are more promising as stimuli-responsive materials [219]. Beside the fact that heparin has shown to accelerate gastric ulcer healing, chitosan-heparin nanocomplex for the delivery of berberine, a natural isoquinoline used in traditional Eastern medicine to treat gastro-enteritis, showed a pH-dependent drug release which was up to 19% of the initial amount at pH 1.2, ~ 10% at pH 6 and ~ 50% at pH 7 due to collapse of the complex [218]. Chitosan-gold nanoparticles of size below 50 nm were used to stabilize the surface of negatively charged liposomes comprising L-α-phosphatidylcholine and 1,2-dioleoyl-*sn*-glycero-3-phosphate and prevent rapid liposome aggregation and fusion [220]. Thus, the coated liposomes were able to release only 10% of doxycycline pH 1.2 and a release up to 90% at pH 7.4 within 24 h and to fuse with the *H. pylori* only at pH 7.4. Moreover, only the coated doxycycline-loaded liposomes were able to inhibit the bacterial growth completely respect to the plain drug or the empty coated liposomes [220].

Site-specific drug targeting

Formulations of higher complexity comprise a functionalization of the surface which allows specific interaction directly with *H. pylori* surface. With this purpose, Umamaheshwari *et al*. [221] anchored on the surface of polyvinyl alcohol beads for the release of acetohydroxamic acid a lipid bilayer of phosphatidylethanolamine (PE) with the aim of plug-and-seal specific receptor on *H. pylori* surface. Besides their ability to inhibit the bacterial growth completely

and to be more stable than normal liposomes, they also showed to prevent the adhesion of *H. pylori* to a cell monolayer and gastric tissue section [221]. The concept of plug-and-seal as an approach to prevent bacterial infection is the topic of intense research of discovery of new potential inhibitors (e.g., polysaccharide) and their usage as anti-adhesive preparation [222].

Due to the presence of adhesines on *H. pylori* surface able to recognize fucose-bearing antigens on the epithelial/mucosal surface, fucose has been introduced in nanoformulation as targeting moiety. Ramteke *et al*. used a carbodiimide method to conjugate covalently fucose to chitosan [223]. This conjugate was used to prepare chitosan-glutamic acid nanoparticles for the concomitant delivery of amoxicillin, clarithromycin and omeprazole which were able to eradicate *H. pylori* from Swiss albino mice respect the unconjugated chitosan-glutamate nanoparticles or plain drugs [223].

Lin *et al*. [197] combined the fucose-conjugated chitosan and genipin-crosslinking technology to formulate a chitosan-heparin nanocomplex for the delivery of amoxicillin. Such a formulation was obviously more effective in eradicating *H. pylori* from infected mice than plain amoxicillin due to the most efficient interaction with bacterial receptor recognizing fucose and also to reduce the *H. pylori*-associated gastric inflammation as concluded by histological inspection [197].

More recently, a site-specific chitosan/TPP nanoparticle loaded with amoxicillin was produced by conjugating chitosan with the ureidododecanoid acid, introducing, therefore, a moiety recognized by the urease-transporter protein present on *H. pylori* surface [224]. The ureido-modified nanoparticles were superior in inhibiting the bacterial growth respect plain amoxicillin and unmodified chitosan/TPP ones. Moreover, such inhibition of the growth was reduced by the addition of competitive substrate urea suggesting that the antibacterial activity is due to a direct delivery of amoxicillin on the bacterial surface as evidenced by flow cytometry analysis, CLSM imaging and OD measurements of bacterial growth [224].

c. In the oral cavity

The oral cavity includes different structures, most important being the lips, the cheeks, the palate, the floor of the mouth and the tongue. The inner surface of the oral cavity is protected by a mucous membrane. The secretion of saliva moisturizes the mucus membrane and form the acquired enamel pellicle at the teeth and is by such very important in order to have a good oral health [225]. Saliva is constantly produced from three major glands and is composed of inorganic ions such as phosphate and calcium as well as organic constituents such as proteins, carbohydrates and lipids. The microflora of the oral cavity is rich and more than 700 different bacteria species can be found here [226]. The pH of a normal healthy mouth is around 6.5-7.5 [227].

The oral cavity can be used for both local drug delivery for treating different infections of the oral mucosa and diseases connected to the teeth such as dental caries and periodontitis in addition to systemic drug delivery via the buccal route.

The greatest challenge when aiming for drug delivery to the oral cavity is the secretion of saliva which could be as high as up to 7 ml/min [228]. Saliva will efficiently flush any foreign substances, also drugs, away. Also the gingival crevicular fluid (GCV) dilutes and flush away substances placed in the periodontal pockets [229]. A mucoadhesive formulation has therefore been proposed in order to prolong the residence time in the oral cavity. However, the mucus layer of the oral cavity has a turnover rate of 12-24 hours [227]. This implies that the residence time in the oral cavity can never be longer than this period of time. In addition, eating, drinking, swallowing and chewing lower this period of time even further. Many new formulations have lately been approved for treating periodontitis [230, 231]. The success of these formulations is due to the use of mucoadhesive polymers enabling the formulation to stay as a reservoir in the periodontal pockets for an extended period of time. However, the periodontal pockets can

perhaps be seen as an easier target than the oral mucosa; when the formulation is placed in the pocket the environmental challenge is more predictable.

Different studies have revealed positively charged materials such as chitosan and positively charged lipids to exert the highest mucoadhesive/bioadhesive properties in the oral cavity [232]. However, toxicity studies have shown that positively charged formulations seem to be more toxic than their negative counterparts [233]. This complicates the picture since the formulations with the highest degree of mucoadhesion also seem to give the highest toxicity.

A formulation, especially a nano- or micro formulation, placed in the oral cavity should also be non-reactive towards saliva. Saliva is composed of globular proteins that can react with the formulation. A study by Nguyen *et al.* showed that positively charged liposomes reacted strongly with saliva. Also some of the negatively charged liposome reacted dependent on the type of negatively charged lipid used [234]. However, when the liposomes were coated with the biopolymer pectin, the interaction disappeared [235].

d. Colorectal drug delivery

The colorectal mucosa can be regarded as an optimal site for drug delivery, following oral or rectal (e.g. suppositories, enemas etc.) administration. For instance, the colon mucosa contains less digestive enzymes and therefore harbors reduced proteolytic activity than the mucosa of the stomach or small intestine [236]. Thus, especially small peptides or proteins can be absorbed in higher concentrations due to less degradation. Furthermore, colonic bacteria can be exploited for the metabolism of prodrugs into effective metabolites [237].

These characteristics rendered the colorectal mucosa an important target for systemic drug delivery. Furthermore, local administration of various drugs poses an important basis for the treatment of various colorectal diseases such as infectious colitis, inflammatory bowel diseases (IBD) or colorectal cancer. In this concern, it is challenging to optimize the bioavailability of

drugs with a maximum concentration at the absorbing/inflamed site for a prolonged time together with minimal systemic side effects. Micro-and nanoformulations can be designed either to increase the stability of drugs, to optimize the ratio between the loaded amount and the loading volume, and to perform a passive or active delivery in the colorectal mucosa [238]. Charged biocompatible polymers (e.g polysaccharides) are able to interact with both healthy and inflamed mucosa by virtue of their numerous charges, high molecular weight and chain flexibility. In addition, some of them are characterized by pH-dependent properties (e.g solubility), which render them optimal materials for the generation of micro-and nanoformulations with mucoadhesive properties. This further enables a prolonged contact with the mucosa and favors cellular uptake. Additionally, pH sensitivity protects the drug from the acidic environment of the stomach and allows its release in lower GI regions [239].

Thus, mucoadhesive strategies have been exploited to improve systemic or local drug administration in various ways. In the subsequent sections, we will discuss challenges and strategies for colorectal targeting of mucoadhesive formulations and possible medical indications.

Micro- and nanoformulations for colorectal delivery

Regarding the design of micro-and nanoformulation for colorectal application, large attention has been posed to the use of polysaccharides due to numerous advantages namely i) their susceptibility to degradation by glycosidases produced by the intestinal microflora, which avoids their accumulation and favor drug release, ii) their ability to interact with the mucosa, which favors prolonged contact with the absorption site, and in special cases iii) to function as absorption enhancer or as promoter of wound healing [240].

Almost three decades ago, the ability of chitosan to promote drug absorption through the intestinal epithelium and also other mucosas such as the nasal one was observed in models of

cell monolayers [241]. Since that, chitosan has been intensively used to generate challenging formulations for the oral delivery drugs, e.g.insulin and others, in form of TPP-crosslinked nanoparticles [242] alone or entrapped into a liposome structure [243], in combination with alginate [244, 245], gum arabic [246], hyaluronic acid [247], lecithin [248] among many others. Chitosan-based formulations can be tailored to specific purposes addressing for instance pH-sensitivity by using a multi-ion crosslinking strategy based on TPP, SO_4^{2-} and Mg^{2+} as recently reported by Lin *et al.*,[249] or adding selective interaction with globet cells by chemically modifying N-trimethyl chitosan chloride with a CSK targeting peptide [250]. Other authors use chitosan and albumin to coat pH-sensitive insulin-loaded alginate/dextran sulfate nanoparticles and investigated the delivery of insulin in presence or absence of inhibitors of permeability [251]. Mucoadhesive formulations have not been proposed only for incorporation and delivery of drugs for already established treatment (e.g insulin, steroids, IBD therapeutics [252]) as will be discussed in the following section, but also for new therapeutic strategies such as delivery of antisense RNA sequences (e.g siRNA technology)[253].

Mucoadhesive formulations for the treatment of colorectal inflammation and cancer

As previously discussed, mucoadhesive formulations have been evaluated in order to optimize the local treatment of colorectal diseases. Currently available studies were mainly aiming at an improved treatment of infectious colitis, inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC) and colorectal cancer (CRC).

Regarding infectious colitis, mucoadhesive formulations have been developed for the treatment of *Clostridium difficile* infection (CDI). *Clostridium difficile* is a toxin-producing, gram-positive bacterium that causes mild to severe colitis, frequently following previous treatment with antibiotics. Symptoms range from asymptomatic carriage to severe disease with toxic megacolon and standard treatment includes antibiotics such as vancomycin or metronidazole. As infection reoccurs in about 10 to 40 percent of cases following initially successful therapy,

there is a huge demand for improved therapeutics. In order to increase colonic delivery of vancomycin for the treatment of CDI, Bigucci *et al*. created vancomycin-containing chitosan/pectin polyelectrolyte complexes [254]. These complexes show pH-dependent swelling and drug release together with colonic mucoadhesion, which suggest superior drug delivery in comparison to standard formulations. However, *in vivo* data supporting this concept are missing so far.

In addition to infectious colitis, IBD also pose an interesting target for mucoadhesive drug formulations. Both CD and UC result in a chronic relapsing inflammation of the gastrointestinal tract that leads to severe complaints including diarrhea, abdominal pain, fever and rectal bleeding in affected patients. Whereas UC is restricted to the large intestine, CD can affect every part of the gastrointestinal tract including the large intestine. Treatment of IBD includes various immunomodulatory approaches including salicylates, steroids, immunosuppressives and biologicals such as anti-TNF therapeutics depending on the severity of disease activity. Especially mild active disease is frequently treated with locally administrated drugs such as salicylates and steroids as oral formulations, enemas or suppositories. Especially oral drug delivery harbours the challenge of selective targeting of the colorectal region in patients with Crohn's colitis or ulcerative colitis. In this regard, mucoadhesive strategies have been evaluated to improve colorectal drug delivery for IBD treatment. Similar to colorectal drug targeting for systemic therapy, mucoadhesive formulations containing chitosan and/or alginate particles have been exploited to deliver standard IBD therapeutics such as 5-aminosalicylic acid [255] or prednisolone to the large intestine [256, 257]. Interestingly, negatively charged liposomes show an improved drug delivery in comparison to the free drug solution in experimental models of colitis, possibly due to an increased adhesion of negatively charged liposomes to the inflamed mucosa. Again, most of these data have been generated either *in vitro* or *in vivo* with preclinical models of intestinal

inflammation. Thus, a proof for a transfer of these strategies for the treatment of human diseases is still missing.

e. Vaginal drug delivery

The vaginal tract has a relatively large surface area of 60 cm^2 and a rich blood supply. The pH of the vaginal tract is controlled by the bacteria *Lactobacillus* converting glycogen and carbohydrates to lactic acid. The pH varies between 4 and 5 depending on the menstrual cycle [258]. There are no secreting glands in the vagina and the amount of fluid is therefore sparse i.e. around 6g [259]. Only small amount of additional liquid can be held without starting to leak out. The vaginal fluid consists of inorganic and organic salts, mucin, proteins, carbohydrates, urea and fatty acids. The vaginal fluid acts as a protecting barrier and consists of different antimicrobial substances [260]. The surface of the vaginal tract is covered by a mucous membrane. The mucin-layer consists of two different types of mucins; cell-associated mucin and secreted mucin forming the outer layer [261]. The secreted mucin has a rapid turnover and can trap foreign particles which will then be efficiently cleared away.

The vaginal tract can in principle be used for both systemic and local delivery of drugs, where local drug delivery for combating for instance fungal, bacterial or viral infections has been the interest of many studies.

There are many challenges related to achieving local drug delivery to the vaginal tract. The formulation must be able withstand changes in the pH, release the drug in the small amount of fluid present and also be able to penetrate the mucus layer before cleared away by the self-cleaning action of vagina. In addition the formulation must not leak out of the vagina [261].

A mucoadhesive formulation will have the possibility of enhancing the time the formulation stays in the vagina, however; often the formulation also need to penetrate the mucus layer in order to achieve the desired effect. This could be complicated if the formulation stick too well

to the mucus layer. In addition, mucoadhesive particles may change the protecting properties of the mucin-layer, letting both the drug and pathogens permeate the mucus barrier [262]. The size of the particle seems to be important in order to be able to penetrate the mucus-layer. Nanoparticles have therefore been proposed to be a promising formulation in order to achieve local vaginal drug delivery [263]. A new approach for obtaining local vaginal drug delivery is muco-resistant nanoparticles such as polyethylene glycol (PEG) coated particles [264]. These particles can diffuse faster through the mucus-layer than mucoadhesive particles. The size of the particles should be between 200-500 nm.

f. Nasal delivery

Since the pioneering studies on chitosan as a nasal penetration enhancer [265], the nasal route has become an attractive option for transmucosal drug delivery, especially for protein/peptide drugs. The nasal mucosa consists of epithelial cells underlined with rich vascularity that provides direct entry of the drug into systemic circulation via passive diffusion and eventual rapid onset of the pharmacological effect. The area available for drug absorption is relatively large but still limited and not easily available. Similarly to the intestinal mucosa, nasal mucosa is monostratified and characterized by the presence of tight junctions and by an abundant mucus secretion. Approximately 1.5–2.0 litres of mucus are secreted daily by goblet cells and serous glands within the nasal cavity, known to contain ca. 1% of proteins, including several proteases[266]. Moreover, mucociliary clearance is a local defence mechanism with regard to respiratory airways that tends to remove foreign bodies (namely bacteria) including formulations from the mucosal site.

Due to the above anatomical and physiological constraints, administration of drugs with the aim of systemic absorption inevitably requires the use of absorption promoters, typically

chemical penetration enhancers, but also of enzyme inhibitors, to escape peptidase action and the use of mucoadhesive formulations to increase the duration and intimacy of contact with the nasal mucosa. Finally, a prerequisite for nasally applied formulations is that it does not interfere with normal nasal functioning. Since nasal mucosa is prone to be damaged by penetration enhancers, which would impair its functions, special attention should be given to the cytotoxicity of absorption promoters and the reversibility of their effect on the nasal membrane should be ascertained.

Besides the vulnerability and the above limitations, the nasal route can be easily managed with an appropriate mucoadhesive formulation for the administration of poorly absorbable peptide/protein drugs[267] or by avoiding hepatic first-pass effect. The use of chitosan and chitosan derivatives, well-known multifunctional polymers, certainly has represented a valid strategy to overcome such limitations. [268, 269] The mucoadhesion properties of chitosan can be exploited also in nasal administration of vaccines.[270] Due to the rapid entry to blood circulation, the nasal route is also promising in the management of crisis situations and intense acute pain, such as heart attack, hypoglycaemia, seizure, severe nausea and vomiting, or breakthrough cancer pain.

Finally, nasal mucosa is also the target for locally applied, locally acting products intended for the treatment of various upper respiratory syndromes such as flu-like symptoms (common cold) and allergic rhinitis. These pathological conditions are characterised by an abundant mucus production accompanied by headache and discomfort. Those symptoms might be caused by viral/bacterial infections that are common pathologies affecting both adults and, more frequently, children of school age. They might also be caused by acute and chronic reactions to allergens.

Pharmacological treatment requires oral administration of anti-inflammatory drugs (aspirin, paracetamol, NSAIDs) and antibiotics as well as local treatment, through sprays or aerosol, with corticosteroids and antihistamines, which is likely to produce side effects such as mucosal dryness and secondary fungal infections. Given that mucus overproduction is one of the most fastidious symptoms, likely to be severe in children, a valuable approach could be to exploit the well-known interaction between chitosan and mucins to obtain a mucolytic effect, thus counteracting the mucus excess. The supposed mechanism of action is the physical interaction between the negatively charged mucin macromolecules and the positively charged side groups of chitosan. The approach has led to the development of a liquid nasal spray, having low viscosity to ensure sprayability, intended to reduce or eliminate the excess of mucous liquid production in rhino-faryngeal diseases (Figure 10.1).

Figure 10.1 Nasal spray

The commercial formulation, developed as a medical device and named Captomucil ® contains a specially devised chitosan grade sourced from fungi having an average MW of 15000 Da and a deacetylation degree of 70%, soluble at neutral pH (6-7) and lacking any irritancy towards nasal mucosa (Patent App. N. MI2014A000825) and likely to produce a mucolytic effect even at very low concentrations. To assess the functionality of the medical device, a rheological

approach was used, based on two different techniques [158, 271] to measure the interaction between chitosan and mucin. Both techniques imply the use of highly-purified mucin, sourced either from the submascillary cavity of cows or from the stomach of pigs. Two sets of formulations were tested, that differed for the salt contents, since it is known that tonicity has an effect on mucus rheology and on nasal mucociliary clearance [272]. In the first sets of experiment, performed according Rossi S. *et al.*[158], an ipotonic Captomucil® formulation (i.e., without salt addition, with chitosan concentration ranging between 0.15-0.16 w/v), was examined for its capability of reducing the viscosity of the submascillary bovine mucin solution in the concentration range 0.5-6% w/w of mucin. The liquid formulation was mixed with mucin solution at different concentrations (see Figure 10.2) in a volumetric ratio mimicking the physiological one. Blank samples (mucin or formulation) were also prepared by diluting with distilled water appropriate volumes of either mucin dispersion (mucin blank) or liquid formulation (formulation blank).

Viscometric measurements were effected at 37°C on each and every set of samples (mucin blank, formulation blank and mucin-formulation mixture) with a rotational rheometer. The rheological interaction between mucin and chitosan was quantified by means of the rheological synergism parameter $\Delta\eta$ (mPa.s), calculated with equation 3.

$$\Delta\eta = \eta_{mix} - (\eta_{f} + \eta_{muc}) \qquad \text{Eq. 3}$$

Where: η_{mix}=viscosity of the formulation and mucin mixture, η_{f}=viscosity of formulation blank and η_{muc}=viscosity of mucin blank. The results (Figure 10.2) showed positive rheological synergism values at very low mucin concentration (0.5-% w/w) and low shear rates, whereas at the highest mucin concentration (4-6% w/w) definite negative rheological synergism values were recorded. Since the synergism values observed at low values (0.5-2% w/v) and at low shear rates were affected by a high standard deviation, it was suggested that the method were not sensitive enough at very low concentrations

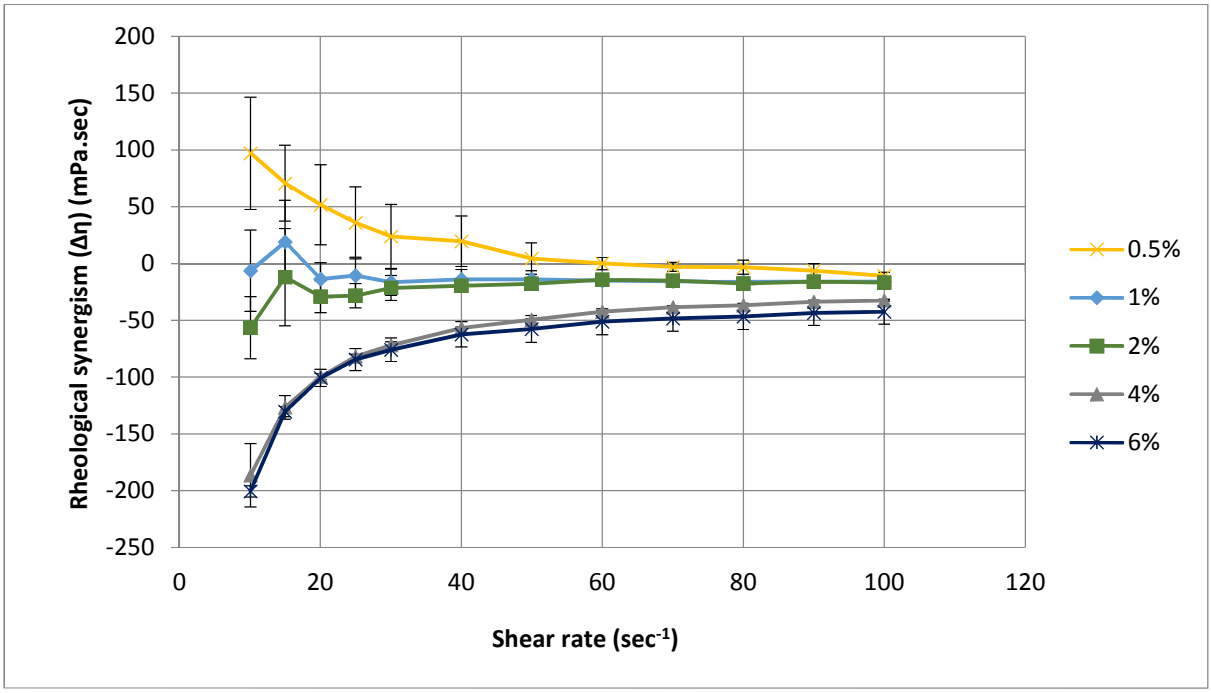

Figure 10.2 Rheological synergism as a function of shear rate evaluated for five formulation-mucin mixtures (mean values +/- sd; n=3)

By contrast, the definite negative synergism values observed at high concentrations (4-6% w/v) indicated that, in those conditions, chitosan produces the maximum interaction with mucin macromolecules. In other words, a massive precipitation of mucus should occur when in presence of a very viscous mucus [273]. To deal with the limited sensitivity of the method at low concentrations, in the second sets of experiments performed according to [271], three Captpmucil® formulations having different contents in chitosan and sodium chloride (chitosan concentration was 0.25, 0.45 and 0.16% w/v, NaCl concentration was 2.2, 2.2 and 0.40 w/v%, respectively) were examined for dynamic viscosity using a falling sphere microviscometer.

Using this method it is possible to work at very low shear rate with precision and probe differences in rheological behavior with a very high sensitivity. The deviation of the viscosity of the mixture mucin/formulation from the sum of the viscosity of mucin solution plus the viscosity of the formulation represents the rheological synergism that may be either positive or negative depending on the sign of the deviation. The method allowed to explore

mucin/formulation ratio much lower than the other method ranging between 0.1 to 1.0 The results obtained for the 3 formulation tested are given in Figure 10.3.

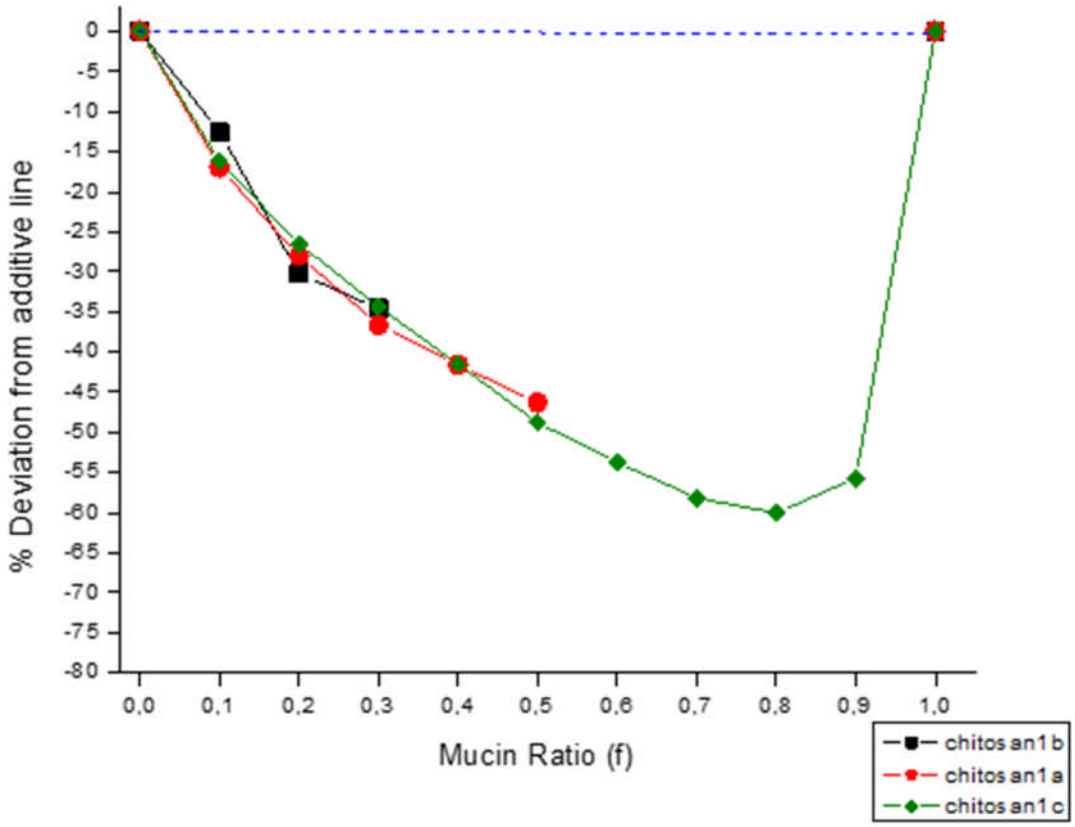

Figure 10.3 Relative deviation (in %) from the additive line (i.e., of no interaction) of the relative viscosity (η_{rel}) of chitosan-based Captomucil® formulations (as in label) in the presence of pig gastric mucin as a function of mucin/formulation mass ratio f (37 °C).

A negative deviation is observed within the whole range of mucin/captomucil ratios tested and the maximum negative deviation, meaning the maximum interaction and mucus precipitation thereof, occurs in the whole range of mucin/formulation ratio for the mixture 1c (green symbols) having the lower Captomucil® concentration and with the lower salt concentration (slightly hypertonic formulation) whereas higher concentrations of Captomucil® and salt in 1a

and 1b (black and red symbols) (hypertonic formulation) caused a weaker interaction specifically not in the whole range of ratios examined.

These results allowed to choose the best concentration of chitosan and salts for the final formulation. Moreover, the results obtained demonstrate that the rheological approaches, appropriately used according to physiological conditions; can be profitably used to measure the precipitation capacity of mucolytic formulations.

11. **Conclusion**

In summary, throughout this review, we present an updated overview of the recent progress on the experimental methods and applications in mucoadhesion research. As illustrated with reference to specific cases, the method of choice to examine the mucoadhesive properties of a given biomaterial (namely a medical device or pharmaceutical formulation) can vary widely. A sounder understanding of the phenomena at play at the molecular level, that influence the interactions of mucin and mucus with a large diversity of materials is likely to contribute to the development of innovative applications on a more rational basis. Novel methods currently under development and that have not been addressed in this review include the use of *in vitro* cell culture or biomicrofluidics approaches.

12. **References**

[1] V. Grabovac, D. Guggi, A. Bernkop-Schnurch, *Advanced Drug Delivery Reviews* **2005**, *57*, 1713.

[2] A. B. Khan, R. S. Thakur, *Current Drug Delivery* **2014**, *11*, 112.

[3] R. Shaikh, T. R. Raj Singh, M. J. Garland, A. D. Woolfson, R. F. Donnelly, *Journal of pharmacy & bioallied sciences* **2011**, *3*, 89.

[4] D. S. Jones, A. D. Woolfson, A. F. Brown, W. A. Coulter, C. McClelland, C. R. Irwin, *Journal of Controlled Release* **2000**, *67*, 357.

[5] J. D. Smart, *Advanced Drug Delivery Reviews* **2005**, *57*, 1556.

[6] L. M. Ensign, R. Cone, J. Hanes, *Advanced Drug Delivery Reviews* **2012**, *64*, 557.

[7] B. C. Tang, M. Dawson, S. K. Lai, Y. Y. Wang, J. S. Suk, M. Yang, P. Zeitlin, M. P. Boyle, J. Fu, J. Hanes, *Proceedings of the National Academy of Sciences of the United States of America* **2009**, *106*, 19268.

[8] R. A. Cone, *Adv Drug Deliver Rev* **2009**, *61*, 75.

[9] M. E. V. Johansson, H. Sjövall, G. C. Hansson, *Nature reviews Gastroenterology Hepatology* **2013**.

[10] J. L. McAuley, S. K. Linden, C. W. Png, R. M. King, H. L. Pennington, S. J. Gendler, T. H. Florin, G. R. Hill, V. Korolik, M. A. McGuckin, *J.Clin.Invest* **2007**, *117*, 2313.

[11] Y. H. Sheng, S. Triyana, R. Wang, I. Das, K. Gerloff, T. H. Florin, P. Sutton, M. A. McGuckin, *Mucosal Immunology* **2013**, *6*, 557.

[12] A. P. Corfield, *Biochimica Et Biophysica Acta-General Subjects* **2015**, *1850*, 236.

[13] S. A. Rayment, B. Liu, G. D. Offner, F. G. Oppenheim, R. F. Troxler, *Journal of dental research* **2000**, *79*, 1765.

[14] K. C. Kim, *Pulmonary pharmacology & therapeutics* **2012**, *25*, 415.

[15] D. J. Thornton, K. Rousseau, M. A. McGuckin, *Annu.Rev.Physiol* **2008**, *70*, 459.

[16] I. K. Gipson, *Frontiers in bioscience : a journal and virtual library* **2001**, *6*, D1245.

[17] S. K. Lai, Y. Y. Wang, J. Hanes, *Advanced Drug Delivery Reviews* **2009**, *61*, 158.

[18] A. Macierzanka, A. R. Mackie, B. H. Bajka, N. M. Rigby, F. Nau, D. Dupont, *Plos One* **2014**, *9*, e95274.

[19] A. N. Round, M. Berry, T. J. McMaster, A. P. Corfield, M. J. Miles, *Journal of Structural Biology* **2004**, *145*, 246.

[20] C. Atuma, V. Strugala, A. Allen, L. Holm, *American Journal of Physiology-Gastrointestinal and Liver Physiology* **2001**, *280*, G922.

[21] M. E. Johansson, M. Phillipson, J. Petersson, A. Velcich, L. Holm, G. C. Hansson, *Proc.Natl.Acad.Sci.U.S.A* **2008**, *105*, 15064.

[22] M. E. V. Johansson, J. M. H. Larsson, G. C. Hansson, *Proceedings of the National Academy of Sciences of the United States of America* **2011**, *108*, 4659.

[23] B. H. Bajka, N. M. Rigby, K. Cross, A. Macierzanka, A. R. Mackie, *Colloids and Surfaces B: Biointerfaces* **2015**, *135*, 73.

[24] S. S. Olmsted, J. L. Padgett, A. I. Yudin, K. J. Whaley, T. R. Moench, R. A. Cone, *Biophysical Journal* **2001**, *81*, 1930.

[25] A. N. Round, N. M. Rigby, A. Garcia de la Torre, A. Macierzanka, E. N. C. Mills, A. R. Mackie, *Biomacromolecules* **2012**, *13*, 3253.

[26] A. Macierzanka, N. M. Rigby, A. P. Corfield, N. Wellner, F. Böttger, E. N. C. Mills, A. R. Mackie, *Soft Matter* **2011**, *7*, 8077.

[27] "*Mucins Methods and protocols"*, 2012, p. 325.

[28] D. J. Thornton, I. Carlstedt, M. Howard, P. L. Devine, M. R. Price, J. K. Sheehan, *Biochemical Journal* **1996**, *316*, 967.

[29] B. D. Raynal, T. E. Hardingham, D. J. Thornton, J. K. Sheehan, *Biochem J* **2002**, *362*, 289.

[30] R. Mehrotra, D. J. Thornton, J. K. Sheehan, *Biochem J* **1998**, *334 (Pt 2)*, 415.

[31] C. Wickstrom, J. R. Davies, G. V. Eriksen, E. C. Veerman, I. Carlstedt, *Biochem J* **1998**, *334 (Pt 3)*, 685.

[32] S. Rossi, M. C. Bonferoni, G. Lippoli, M. Bertoni, F. Ferrari, C. Caramella, U. Conte, *Biomaterials* **1995**, *16*, 1073.

[33] G. E. Yakubov, A. Papagiannopoulos, E. Rat, R. L. Easton, T. A. Waigh, *Biomacromolecules* **2007**, *8*, 3467.

[34] S. Kirkham, J. K. Sheehan, D. Knight, P. S. Richardson, D. J. Thornton, *Biochemical Journal* **2002**, *361*, 537.

[35] D. J. Thornton, N. Khan, R. Mehrotra, M. Howard, E. Veerman, N. H. Packer, J. K. Sheehan, *Glycobiology* **1999**, *9*, 293.

[36] M. T. Cook, V. V. Khutoryanskiy, *Int J Pharm* **2015**, *495*, 991.

[37] A. C. Groo, F. Lagarce, *Drug discovery today* **2014**, *19*, 1097.

[38] S. P. Authimoolam, T. D. Dziubla, *Polymers* **2016**, *8*.

[39] C. D. Rillahan, J. C. Paulson, *Annual review of biochemistry* **2011**, *80*, 797.

[40] S. M. Muthana, J. C. Gildersleeve, *Cancer biomarkers : section A of Disease markers* **2014**, *14*, 29.

[41] K. Godula, D. Rabuka, K. T. Nam, C. R. Bertozzi, *Angewandte Chemie (International ed. in English)* **2009**, *48*, 4973.

[42] M. Kilcoyne, J. Q. Gerlach, R. Gough, M. E. Gallagher, M. Kane, S. D. Carrington, L. Joshi, *Anal Chem* **2012**, *84*, 3330.

[43] J. Cleary, L. Bromberg, E. Magner, *Langmuir* **2004**, *20*, 9755.

[44] N. V. Efremova, Y. Huang, N. A. Peppas, D. E. Leckband, *Langmuir* **2002**, *18*, 836.

[45] M. Iijima, M. Yoshimura, T. Tsuchiya, M. Tsukada, H. Ichikawa, Y. Fukumori, H. Kamiya, *Langmuir* **2008**, *24*, 3987.

[46] D. Li, H. Yamamoto, H. Takeuchi, Y. Kawashima, *European Journal of Pharmaceutics and Biopharmaceutics* **2010**, *75*, 277.

[47] P. Sriamornsak, N. Wattanakorn, H. Takeuchi, *Carbohydrate Polymers* **2010**, *79*, 54.

[48] C. Taylor, J. P. Pearson, K. I. Draget, P. W. Dettmar, O. Smidsrod, *Carbohydrate Polymers* **2005**, *59*, 189.

[49] M. Boegh, S. G. Baldursdottir, A. Mullertz, H. M. Nielsen, *European Journal of Pharmaceutics and Biopharmaceutics* **2014**, *87*, 227.

[50] B. T. Burruano, R. L. Schnaare, D. Malamud, *Contraception* **2002**, *66*, 137.

[51] C. V. Duffy, L. David, T. Crouzier, *Acta Biomaterialia* **2015**, *20*, 51.

[52] R. Hamed, J. Fiegel, *Journal of Biomedical Materials Research Part A* **2014**, *102*, 1788.

[53] M. D. Anwarul Hasan, C. F. Lange, M. L. King, *Journal of Non-Newtonian Fluid Mechanics* **2010**, *165*, 1431.

[54] S. P. Authimoolam, A. L. Vasilakes, N. M. Shah, D. A. Puleo, T. D. Dziubla, *Biomacromolecules* **2014**, *15*, 3099.

[55] M. Doellinger, F. Groehn, D. A. Berry, U. Eysholdt, G. Luegmair, *Journal of Speech Language and Hearing Research* **2014**, *57*, S637.

[56] D. J. Hall, O. V. Khutoryanskaya, V. V. Khutoryanskiy, *Soft Matter* **2011**, *7*, 9620.

[57] J. KocevarNared, J. Kristl, J. SmidKorbar, *Biomaterials* **1997**, *18*, 677.

[58] T. J. Sill, H. a. von Recum, *Biomaterials* **2008**, *29*, 1989.

[59] Q. P. Pham, U. Sharma, A. G. Mikos, *Tissue Engineering* **2006**, *12*, 060509065116001.

[60] S. Ramakrishna, M. Zamani, M. P. Prabhakaran, *International Journal of Nanomedicine* **2013**, *8*, 2997.

[61] K. Stephansen, M. García-Díaz, F. Jessen, I. S. Chronakis, H. M. Nielsen, *International Journal of Pharmaceutics* **2015**, *495*, 58.

[62] S.-F. Chou, D. Carson, K. A. Woodrow, *Journal of Controlled Release* **2015**, *220*, 584.

[63] A. Balaji, M. V. Vellayappan, A. A. John, A. P. Subramanian, S. K. Jaganathan, E. Supriyanto, S. I. A. Razak, *RSC Adv.* **2015**, *5*, 57984.

[64] A. Sharma, A. Gupta, G. Rath, A. Goyal, R. B. Mathur, S. R. Dhakate, *Journal of Materials Chemistry B* **2013**, *1*, 3410.

[65] S. Wongsasulak, S. Pathumban, T. Yoovidhya, *Journal of Food Engineering* **2014**, *120*, 110.

[66] C. Dott, C. Tyagi, L. K. Tomar, Y. E. Choonara, P. Kumar, L. C. du Toit, V. Pillay, *Journal of Nanomaterials* **2013**, *2013*, 1.

[67] R. Sridhar, S. Sundarrajan, A. Vanangamudi, G. Singh, T. Matsuura, S. Ramakrishna, *Macromolecular Materials and Engineering* **2014**, *299*, 283.

[68] F. Ding, H. Deng, Y. Du, X. Shi, Q. Wang, *Nanoscale* **2014**, *6*, 9477.

[69] K. Stephansen, M. García-Díaz, F. Jessen, I. S. Chronakis, H. M. Nielsen, *Molecular Pharmaceutics* **2016**, *13*, 748.

[70] H. Seager, *The Journal of pharmacy and pharmacology* **1998**, *50*, 375.

[71] D. C. Aduba, J. A. Hammer, Q. Yuan, W. Andrew Yeudall, G. L. Bowlin, H. Yang, *Acta Biomaterialia* **2013**, *9*, 6576.

[72] L. Xu, N. Sheybani, S. Ren, G. L. Bowlin, W. A. Yeudall, H. Yang, *Pharmaceutical Research* **2015**, *32*, 275.

[73] P. Tonglairoum, T. Ngawhirunpat, T. Rojanarata, S. Panomsuk, R. Kaomongkolgit, P. Opanasopit, *Carbohydrate Polymers* **2015**, *132*, 173.

[74] S. Wongsasulak, N. Puttipaiboon, T. Yoovidhya, *Journal of Food Science* **2013**, *78*, N926.

[75] S. Zong, X. Wang, Y. Yang, W. Wu, H. Li, Y. Ma, W. Lin, T. Sun, Y. Huang, Z. Xie, Y. Yue, S. Liu, X. Jing, *European Journal of Pharmaceutics and Biopharmaceutics* **2015**, *93*, 127.

[76] C. Huang, S. J. Soenen, E. van Gulck, G. Vanham, J. Rejman, S. Van Calenbergh, C. Vervaet, T. Coenye, H. Verstraelen, M. Temmerman, J. Demeester, S. C. De Smedt, *Biomaterials* **2012**, *33*, 962.

[77] W. Samprasit, R. Kaomongkolgit, M. Sukma, T. Rojanarata, T. Ngawhirunpat, P. Opanasopit, *Carbohydrate Polymers* **2015**, *117*, 933.

[78] H. Singh, R. Sharma, M. Joshi, T. Garg, A. K. Goyal, G. Rath, *Artificial Cells, Nanomedicine, and Biotechnology* **2015**, *43*, 263.

[79] P. Tonglairoum, T. Ngawhirunpat, T. Rojanarata, R. Kaomongkolgit, P. Opanasopit, *Colloids and Surfaces B: Biointerfaces* **2015**, *126*, 18.

[80] H. Grewal, S. R. Dhakate, A. K. Goyal, T. S. Markandeywar, B. Malik, G. Rath, *Artificial Cells, Blood Substitutes, and Biotechnology* **2012**, *40*, 146.

[81] C. Huang, S. J. Soenen, E. van Gulck, J. Rejman, G. Vanham, B. Lucas, B. Geers, K. Braeckmans, V. Shahin, P. Spanoghe, J. Demeester, S. C. De Smedt, *Polymers for Advanced Technologies* **2014**, *25*, 827.

[82] Gagandeep, T. Garg, B. Malik, G. Rath, A. K. Goyal, *European Journal of Pharmaceutical Sciences* **2014**, *53*, 10.

[83] E. Zartler, J. Yan, H. Mo, A. Kline, M. Shapiro, *Current Topics in Medicinal Chemistry* **2003**, *3*, 25.

[84] S. Monti, I. Manet, G. Marconi, *Physical Chemistry Chemical Physics* **2011**, *13*, 20893.

[85] S. Guglieri, M. Hricovíni, R. Raman, L. Polito, G. Torri, B. Casu, R. Sasisekharan, M. Guerrini, *Biochemistry* **2008**, *47*, 13862.

[86] G. Uccello-Barretta, F. Balzano, F. Aiello, *Polysaccharides: Bioactivity and Biotechnology* **2015**, 1299.

[87] G. Uccello-Barretta, S. Nazzi, F. Balzano, M. Sansò, *International Journal of Pharmaceutics* **2011**, *406*, 78.

[88] G. Uccello-Barretta, F. Balzano, L. Vanni, M. Sansò, *Carbohydrate Polymers* **2013**, *91*, 568.

[89] M. M. Patel, J. D. Smart, T. G. Nevell, R. J. Ewen, P. J. Eaton, J. Tsibouklis, *Biomacromolecules* **2003**, *4*, 1184.

[90] S. A. Mortazavi, *International Journal of Pharmaceutics* **1995**, *124*, 173.

[91] P. C. Griffiths, P. Occhipinti, C. Morris, R. K. Heenan, S. M. King, M. Gumbleton, *Biomacromolecules* **2010**, *11*, 120.

[92] M. Davidovich-Pinhas, H. Bianco-Peled, *Expert Opinion on Drug Delivery* **2010**, *7*, 259.

[93] P. Sriamornsak, N. Wattanakorn, J. Nunthanid, S. Puttipipatkhachorn, *Carbohydrate Polymers* **2008**, *74*, 458.

[94] F. Saiano, G. Pitarresi, G. Cavallaro, M. Licciardi, G. Giammona, *Polymer* **2002**, *43*, 6281.

[95] J. Xiang, X. Li, *Journal of Applied Polymer Science* **2004**, *94*, 2431.

[96] R. Falahat, E. Williams, M. Wiranowska, R. Toomey, N. Alcantar, *Cancer Research* **2014**, *74*, 5410.

[97] N. A. Peppas, Y. Huang, *Advanced Drug Delivery Reviews* **2004**, *56*, 1675.

[98] J. das Neves, M. F. Bahia, M. M. Amiji, B. Sarmento, *Expert Opinion on Drug Delivery* **2011**, *8*, 1085.

[99] H. Takeuchi, Y. Matsui, H. Sugihara, H. Yamamoto, Y. Kawashima, *International Journal of Pharmaceutics* **2005**, *303*, 160.

[100] H. Takeuchi, J. Thongborisute, Y. Matsui, H. Sugihara, H. Yamamoto, Y. Kawashima, *Advanced Drug Delivery Reviews* **2005**, *57*, 1583.

[101] D. Chen, D. Xia, X. Li, Q. Zhu, H. Yu, C. Zhu, Y. Gan, *International Journal of Pharmaceutics* **2013**, *449*, 1.

[102] B. Fan, Y. Xing, Y. Zheng, C. Sun, G. Liang, *Drug delivery* **2016**, *23*, 238.

[103] K. Tahara, S. Fujimoto, F. Fujii, Y. Tozuka, T. Jin, H. Takeuchi, *Journal of Pharmaceutics* **2013**, *2013*, 1.

[104] G. Uccello-Barretta, F. Balzano, F. Aiello, A. Senatore, A. Fabiano, Y. Zambito, *International Journal of Pharmaceutics* **2014**, *461*, 489.

[105] T. Eshel-Green, H. Bianco-Peled, *Colloids and Surfaces B: Biointerfaces* **2016**, *139*, 42.

[106] T. Pettersson, A. Dedinaite, *Journal of Colloid and Interface Science* **2008**, *324*, 246.

[107] L. Joergensen, B. Klosgen, A. C. Simonsen, J. Borch, E. Hagesaether, *International Journal of Pharmaceutics* **2011**, *411*, 162.

[108] M. P. Deacon, S. McGurk, C. J. Roberts, P. M. Williams, S. J. B. Tendler, M. C. Davies, S. S. Davis, S. E. Harding, *Biochemical Journal* **2000**, *348*, 557.

[109] E. Di Cola, G. E. Yakubov, T. A. Waigh, *Biomacromolecules* **2008**, *9*, 3216.

[110] P. Georgiades, E. di Cola, R. K. Heenan, P. D. A. Pudney, D. J. Thornton, T. A. Waigh, *Biopolymers* **2014**, *101*, 1154.

[111] Y. Watanabe, Y. Inoko, *J Appl Crystallogr* **2007**, *40*, S209.

[112] T. A. Waigh, A. Papagiannopoulos, A. Voice, R. Bansil, A. P. Unwin, C. D. Dewhurst, B. Turner, N. Afdhal, *Langmuir* **2002**, *18*, 7188.

[113] S. K. Lai, Y. Y. Wang, D. Wirtz, J. Hanes, *Adv Drug Deliv Rev* **2009**, *61*, 86.

[114] J. P. Celli, B. S. Turner, N. H. Afdhal, R. H. Ewoldt, G. H. McKinley, R. Bansil, S. Erramilli, *Biomacromolecules* **2007**, *8*, 1580.

[115] R. Bansil, H. E. Stanley, J. T. Lamont, *Annual Review of Physiology* **1995**, *57*, 635.

[116] X. Cao, R. Bansil, K. R. Bhaskar, B. S. Turner, J. T. LaMont, N. Niu, N. H. Afdhal, *Biophysical Journal* **1999**, *76*, 1250.

[117] Z. N. Hong, B. Chasan, R. Bansil, B. S. Turner, K. R. Bhaskar, N. H. Afdhal, *Biomacromolecules* **2005**, *6*, 3458.

[118] A. Maleki, G. Lafitte, A. L. Kjoniksen, K. Thuresson, B. Nystrom, *Carbohyd Res* **2008**, *343*, 328.

[119] K. R. Bhaskar, D. H. Gong, R. Bansil, S. Pajevic, J. A. Hamilton, B. S. Turner, J. T. LaMont, *Am J Physiol* **1991**, *261*, G827.

[120] S. Lee, M. Muller, K. Rezwan, N. D. Spencer, *Langmuir* **2005**, *21*, 8344.

[121] I. A. Sogias, A. C. Williams, V. V. Khutoryanskiy, *Biomacromolecules* **2008**, *9*, 1837.

[122] B. Menchicchi, J. P. Fuenzalida, A. Hensel, M. J. Swamy, L. David, C. Rochas, F. M. Goycoolea, *Biomacromolecules* **2015**, *16*, 924.

[123] C. Woertz, M. Preis, J. Breitkreutz, P. Kleinebudde, *European Journal of Pharmaceutics and Biopharmaceutics* **2013**, *85*, 843.

[124] S. Rossi, M. C. Bonferoni, F. Ferrari, M. Bertoni, C. Caramella, *European Journal of Pharmaceutical Sciences* **1996**, *4*, 189.

[125] E. Hagesaether, M. Hiorth, S. A. Sande, *European Journal of Pharmaceutics and Biopharmaceutics* **2009**, *71*, 325.

[126] M. Bogataj, T. Vovk, M. Kerec, A. Dimnik, I. Grabnar, A. Mrhar, *Biological & Pharmaceutical Bulletin* **2003**, *26*, 743.

[127] D. S. Jones, A. D. Woolfson, A. F. Brown, *International Journal of Pharmaceutics* **1997**, *151*, 223.

[128] H. Hägerström, K. Edsman, *Journal of Pharmacy and Pharmacology* **2001**, *53*, 1589.

[129] S. Tamburic, D. Q. M. Craig, *European Journal of Pharmaceutics and Biopharmaceutics* **1997**, *44*, 159.

[130] I. A. Sogias, A. C. Williams, V. V. Khutoryanskiy, *International Journal of Pharmaceutics* **2012**, *436*, 602.

[131] A. P. Sam, J. T. M. van den Heuij, J. J. Tukker, *International Journal of Pharmaceutics* **1992**, *79*, 97.

[132] J. D. Smart, *International Journal of Pharmaceutics* **1991**, *73*, 69.

[133] C. E. Kast, A. Bernkop-Schnürch, *Biomaterials* **2001**, *22*, 2345.

[134] K. G. H. Desai, T. M. P. Kumar, *Aaps Pharmscitech* **2004**, *5*.

[135] H. S. Chng, H. Park, P. Kelly, J. R. Robinson, *Journal of Pharmaceutical Sciences* **1985**, *74*, 399.

[136] R. Gurny, J.-M. Meyer, N. A. Peppas, *Biomaterials* **1984**, *5*, 336.

[137] Y. B. Huang, W. Leobandung, A. Foss, N. A. Peppas, *Journal of Controlled Release* **2000**, *65*, 63.

[138] F. Ferrari, M. Bertoni, S. Rossi, M. C. Bonferoni, C. Caramella, M. J. Waring, M. E. Aulton, *Drug Dev Ind Pharm* **1996**, *22*, 1223.

[139] D. Patel, J. R. Smith, A. W. Smith, N. Grist, P. Barnett, J. D. Smart, *International Journal of Pharmaceutics* **2000**, *200*, 271.

[140] H. Takeuchi, J. Thongborisute, Y. Matsui, H. Sugihara, H. Yamamoto, Y. Kawashima, *Adv Drug Deliv Rev* **2005**, *57*, 1583.

[141] A. Fuongfuchat, A. M. Jamieson, J. Blackwell, T. A. Gerken, *Carbohydrate Research* **1996**, *284*, 85.

[142] S. Rossi, F. Ferrari, M. C. Bonferoni, C. Caramella, *European Journal of Pharmaceutical Sciences* **2000**, *10*, 251.

[143] M. M. Patel, J. D. Smart, T. G. Nevell, R. J. Ewen, P. J. Eaton, J. Tsibouklis, *Biomacromolecules* **2003**, *4*, 1184.

[144] M. P. Deacon, S. S. Davis, R. J. White, H. Nordman, I. Carlstedt, N. Errington, A. J. Rowe, S. E. Harding, *Carbohydrate Polymers* **1999**, *38*, 235.

[145] I. Fiebrig, Davis, S.S., Harding, S.E., "Method used to develop drug delivery systems: bioadhesion in the gastrointestinal tract", in *Biopolymer mixtures*, S.E. Harding, Hill,S.E., Mitchell,J.R., Ed., Nottingham University Press, Nottingham, 1995, p. 737.

[146] S. E. Harding, *Trends in Food Science & Technology* **2006**, *17*, 255.

[147] S. Chayed, F. M. Winnik, *European Journal of Pharmaceutics and Biopharmaceutics* **2007**, *65*, 363.

[148] W. Chaiyasan, S. P. Srinivas, W. Tiyaboonchai, *J Ocul Pharmacol Th* **2013**, *29*, 200.

[149] Y. Bin Choy, J. H. Park, M. R. Prausnitz, *J Phys Chem Solids* **2008**, *69*, 1533.

[150] Y. Miyazaki, K. Ogihara, S. Yakou, T. Nagai, K. Takayama, *International Journal of Pharmaceutics* **2003**, *258*, 21.

[151] I. Bravo-Osuna, C. Vauthier, A. Farabollini, G. F. Palmieri, G. Ponchel, *Biomaterials* **2007**, *28*, 2233.

[152] J. Schmidgall, E. Schnetz, A. Hensel, *Planta Med* **2000**, *66*, 48.

[153] J. Schmidgall, A. Hensel, *Int J Biol Macromol* **2002**, *30*, 217.

[154] K. Park, J. R. Robinson, *International Journal of Pharmaceutics* **1984**, *19*, 107.

[155] O. Lieleg, I. Vladescu, K. Ribbeck, *Biophys J* **2010**, *98*, 1782.

[156] E. E. Hassan, J. M. Gallo, *Pharm Res* **1990**, *7*, 491.

[157] S. J. List, B. P. Findlay, G. G. Forstner, J. F. Forstner, *Biochemical Journal* **1978**, *175*, 565.

[158] S. Rossi, F. Ferrari, M. C. Bonferoni, C. Caramella, *European Journal of Pharmaceutical Sciences* **2001**, *12*, 479.

[159] S. Rossi, M. C. Bonferoni, G. Lippoli, M. Bertoni, F. Ferrari, C. Caramella, U. Conte, *Biomaterials* **1995**, *16*, 1073.

[160] F. Madsen, K. Eberth, J. D. Smart, *Biomaterials* **1998**, *19*, 1083.

[161] C. Taylor, J. P. Pearson, K. I. Draget, P. W. Dettmar, O. Smidsrød, *Carbohydrate Polymers* **2005**, *59*, 189.

[162] F. M. Goycoolea, E. R. Morris, M. J. Gidley, *Carbohydrate Polymers* **1995**, *28*, 351.

[163] D. J. McClements, *Biotechnol Adv* **2006**, *24*, 621.

[164] N. K. Howell, "Synergism and interactions in mixed protein systems", in *Biopolymer mixtures*, S.E. Harding, Hill,S.E., Mitchell,J.R., Ed., Notthingam University Press, Notthingam, 1995, p. 329.

[165] E. R. Morris, "Polysaccharides synergism: more question than answers?", in *Biopolymer Mixtures*, S.E. Harding, Hill,S.E., Mitchell,J.R., Ed., Nottingham University Press, Nottingham, 1995, p. 247.

[166] S. A. Mortazavi, B. G. Carpenter, J. D. Smart, *International Journal of Pharmaceutics* **1993**, *94*, 195.

[167] C. Woertz, M. Preis, J. Breitkreutz, P. Kleinebudde, *European Journal of Pharmaceutics and Biopharmaceutics* **2013**, *85*, 843.

[168] B. Menchicchi, J. P. Fuenzalida, K. B. Bobbili, A. Hensel, M. J. Swamy, F. M. Goycoolea, *Biomacromolecules* **2014**, *15*, 3550.

[169] G. Sandri, S. Rossi, M. C. Bonferoni, F. Ferrari, M. Mori, C. Caramella, *Journal of Drug Delivery Science and Technology* **2012**, *22*, 275.

[170] G. Sandri, S. Rossi, F. Ferrari, M. C. Bonferoni, N. Zerrouk, C. Caramella, *Journal of Pharmacy and Pharmacology* **2004**, *56*, 1083.

[171] E. Szymańska, K. Winnicka, A. Amelian, U. Cwalina, *Chemical and Pharmaceutical Bulletin* **2014**, *62*, 160.

[172] M. Bayarri, N. Oulahal, P. Degraeve, A. Gharsallaoui, *Journal of Food Engineering* **2014**, *131*, 18.

[173] Y. Li, W. Yokoyama, J. Wu, J. Ma, F. Zhong, *RSC Adv.* **2015**, *5*, 105844.

[174] M. A. Güler, M. K. Gök, A. K. Figen, S. Özgümüş, *Applied Clay Science* **2015**, *112-113*, 44.

[175] C. S. Kolli, I. Pather, "Characterization Methods for Oral Mucosal Drug Delivery", in *Oral Mucosal Drug Delivery and Therapy*, M. Rathbone, S. Senel, and I. Pather, Eds., Springer, 2015, p. 125.

[176] C. Pontier, J. Pachot, R. Botham, B. Lenfant, P. Arnaud, *Journal of Pharmaceutical Sciences* **2001**, *90*, 1608.

[177] E. Hagesaether, E. Christiansen, M. E. Due-Hansen, T. Ulven, *International Journal of Pharmaceutics* **2013**, *452*, 276.

[178] A. Jintapattanakit, V. B. Junyaprasert, T. Kissel, *Journal of Pharmaceutical Sciences* **2009**, *98*, 4818.

[179] S. Chen, Y. Cao, L. R. Ferguson, Q. Shu, S. Garg, *J. Microencapsul.* **2013**, *30*, 103.

[180] M. I. Adamczak, E. Hagesaether, G. Smistad, M. Hiorth, *International Journal of Pharmaceutics* **2016**, *498*, 225.

[181] P. Georgiades, P. D. A. Pudney, D. J. Thornton, T. A. Waigh, *Biopolymers* **2014**, *101*, 366.

[182] S. K. Lai, D. E. O'Hanlon, S. Harrold, S. T. Man, Y. Y. Wang, R. Cone, J. Hanes, *Proceedings of the National Academy of Sciences of the United States of America* **2007**, *104*, 1482.

[183] S. K. Lai, Y. Y. Wang, K. Hida, R. Cone, J. Hanes, *Proc.Natl.Acad.Sci.U.S.A* **2010/1/12**, *107*, 598.

[184] A. R. Mackie, A. Macierzanka, K. Aarak, N. M. Rigby, R. Parker, G. A. Channell, S. E. Harding, B. H. Bajka, *Food Hydrocolloids* **2016**, *52*, 749.

[185] J. Kirch, A. Schneider, B. Abou, A. Hopf, U. F. Schaefer, M. Schneider, C. Schall, C. Wagner, C.-M. Lehr, *Proceedings of the National Academy of Sciences of the United States of America* **2012**, *109*, 18355.

[186] D. Ambort, M. E. V. Johansson, J. K. Gustafsson, H. E. Nilsson, A. Ermund, B. R. Johansson, P. J. B. Koeck, H. Hebert, G. C. Hansson, *Proceedings of the National Academy of Sciences of the United States of America* **2012**, *109*, 5645.

[187] T. L. Cover, M. J. Blaser, *Gastroenterology* **2009**, *136*, 1863.

[188] S. Mishra, *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* **2013**, *32*, 301.

[189] J. Parsonnet, G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, R. K. Sibley, *The New England journal of medicine* **1991**, *325*, 1127.

[190] V. De Francesco, F. Giorgio, C. Hassan, G. Manes, L. Vannella, C. Panella, E. Ierardi, A. Zullo, *Journal of gastrointestinal and liver diseases : JGLD* **2010**, *19*, 409.

[191] P. Malfertheiner, F. Megraud, C. A. O'Morain, J. Atherton, A. T. R. Axon, F. Bazzoli, G. F. Gensini, J. P. Gisbert, D. Y. Graham, T. Rokkas, E. M. El-Omar, E. J. Kuipers, T. E. H. S. Group, *Gut* **2012**, *61*, 646.

[192] P. S. Rajinikanth, B. Mishra, *Journal of controlled release : official journal of the Controlled Release Society* **2008**, *125*, 33.

[193] A. Streubel, J. Siepmann, R. Bodmeier, *Expert Opinion on Drug Delivery* **2006**, *3*, 217.

[194] E. A. Klausner, E. Lavy, M. Friedman, A. Hoffman, *Journal of controlled release : official journal of the Controlled Release Society* **2003**, *90*, 143.

[195] D. Lopes, C. Nunes, M. C. L. Martins, B. Sarmento, S. Reis, *Journal of Controlled Release* **2014**, *189*, 169.

[196] J. K. Patel, M. M. Patel, *Curr Drug Deliv* **2007**, *4*, 41.

[197] Y.-H. Lin, S.-C. Tsai, C.-H. Lai, C.-H. Lee, Z. S. He, G.-C. Tseng, *Biomaterials* **2013**, *34*, 4466.

[198] R. B. Umamaheshwari, S. Ramteke, N. K. Jain, *Aaps Pharmscitech* **2004**, *5*, 60.

[199] A. O. Elzoghby, W. M. Samy, N. A. Elgindy, *Journal of Controlled Release* **2012**, *161*, 38.

[200] J. Wang, Y. Tauchi, Y. Deguchi, K. Morimoto, Y. Tabata, Y. Ikada, *Drug delivery* **2000**, *7*, 237.

[201] S. Ramteke, N. K. Jain, *J Drug Target* **2008**, *16*, 65.

[202] S. Ramteke, N. Ganesh, S. Bhattacharya, N. K. Jain, *J Drug Target* **2008**, *16*, 694.

[203] D. Luo, J. Guo, F. Wang, J. Sun, G. Li, X. Cheng, M. Chang, X. Yan, *Journal of biomaterials science. Polymer edition* **2009**, *20*, 1587.

[204] F. Nogueira, I. C. Goncalves, M. C. Martins, *Acta Biomater* **2013**, *9*, 5208.

[205] R. Fernandez-Urrusuno, P. Calvo, C. Remunan-Lopez, J. L. Vila-Jato, M. J. Alonso, *Pharm Res* **1999**, *16*, 1576.

[206] P. Calvo, C. Remuñán-López, J. L. Vila-Jato, M. J. Alonso, *Colloid and Polymer Science*, *275*, 46.

[207] C. K. S. Pillai, W. Paul, C. P. Sharma, *Progress in Polymer Science* **2009**, *34*, 641.

[208] J. A. Raval, J. K. Patel, M. M. Patel, *Acta pharmaceutica (Zagreb, Croatia)* **2010**, *60*, 455.

[209] A. Portero, C. Remunan-Lopez, M. T. Criado, M. J. Alonso, *J Microencapsul* **2002**, *19*, 797.

[210] R. Hejazi, M. Amiji, *Int J Pharm* **2004**, *272*, 99.

[211] M. Fernandes, I. C. Goncalves, S. Nardecchia, I. F. Amaral, M. A. Barbosa, M. C. Martins, *Int J Pharm* **2013**, *454*, 116.

[212] X. Zhu, D. Zhou, S. Guan, P. Zhang, Z. Zhang, Y. Huang, *Journal of Materials Science: Materials in Medicine* **2012**, *23*, 983.

[213] C.-H. Chang, Y.-H. Lin, C.-L. Yeh, Y.-C. Chen, S.-F. Chiou, Y.-M. Hsu, Y.-S. Chen, C.-C. Wang, *Biomacromolecules* **2010**, *11*, 133.

[214] Y. Miyazaki, K. Ogihara, S. Yakou, T. Nagai, K. Takayama, *International journal of pharmaceutics* **2003**, *258*, 21.

[215] R. B. Umamaheshwari, S. Jain, N. K. Jain, *Drug delivery* **2003**, *10*, 151.

[216] Z. Liu, W. Lu, L. Qian, X. Zhang, P. Zeng, J. Pan, *Journal of Controlled Release* **2005**, *102*, 135.

[217] C.-H. Chang, W.-Y. Huang, C.-H. Lai, Y.-M. Hsu, Y.-H. Yao, T.-Y. Chen, J.-Y. Wu, S.-F. Peng, Y.-H. Lin, *Acta Biomaterialia* **2011**, *7*, 593.

[218] Y. H. Lin, C. H. Chang, Y. S. Wu, Y. M. Hsu, S. F. Chiou, Y. J. Chen, *Biomaterials* **2009**, *30*, 3332.

[219] J. P. Fuenzalida, F. M. Goycoolea, *Current protein & peptide science* **2015**, *16*, 89.

[220] S. Thamphiwatana, V. Fu, J. Zhu, D. Lu, W. Gao, L. Zhang, *Langmuir : the ACS journal of surfaces and colloids* **2013**, *29*, 12228.

[221] R. B. Umamaheshwari, N. K. Jain, *Journal of controlled release : official journal of the Controlled Release Society* **2004**, *99*, 27.

[222] B. Menchicchi, A. Hensel, F. M. Goycoolea, *Current Pharmaceutical Design* **2015**, *21*, 4888.

[223] S. Ramteke, N. Ganesh, S. Bhattacharya, N. K. Jain, *Journal of Drug Targeting* **2009**, *17*, 225.

[224] Z.-W. Jing, Y.-Y. Jia, N. Wan, M. Luo, M.-L. Huan, T.-B. Kang, S.-Y. Zhou, B.-L. Zhang, *Biomaterials* **2016**, *84*, 276.

[225] F. Dost, C. S. Farah, *Australian Dental Journal* **2013**, *58*, 11.

[226] J. A. Aas, B. J. Paster, L. N. Stokes, I. Olsen, F. E. Dewhirst, *Journal of clinical microbiology* **2005**, *43*, 5721.

[227] K. Netsomboon, A. Bernkop-Schnürch, *European Journal of Pharmaceutics and Biopharmaceutics* **2016**, *98*, 76.

[228] J. D. Smart, *Crit Rev Ther Drug Carrier Syst* **2004**, *21*, 319.

[229] N. Jain, G. K. Jain, S. Javed, Z. Iqbal, S. Talegaonkar, F. J. Ahmad, R. K. Khar, *Drug discovery today* **2008**, *13*, 932.

[230] G. Mayor-Subirana, J. Yagüe-García, E. Valmaseda-Castellón, J. Arnabat-Domínguez, L. Berini-Aytés, C. Gay-Escoda, *Medicina oral, patologia oral y cirugia bucal* **2014**, *19*, e192.

[231] X. Song, T. Yaskell, V. Klepac-Ceraj, M. C. Lynch, N. S. Soukos, *Journal of periodontology* **2014**, *85*, 335.

[232] M. I. Adamczak, E. Hagesaether, G. Smistad, M. Hiorth, *Int. J. Pharm.* **2016**, *498*, 225.

[233] G. Smistad, J. Jacobsen, S. A. Sande, *International Journal of Pharmaceutics* **2007**, *330*, 14.

[234] S. Nguyen, M. Hiorth, M. Rykke, G. Smistad, *European Journal of Pharmaceutics and Biopharmaceutics* **2011**, *77*, 75.

[235] S. Nguyen, M. Hiorth, M. Rykke, G. Smistad, *European Journal of Pharmaceutical Sciences* **2013**, *50*, 78.

[236] M. K. Chourasia, S. K. Jain, *Journal of pharmacy & pharmaceutical sciences : a publication of the Canadian Society for Pharmaceutical Sciences, Societe canadienne des sciences pharmaceutiques* **2003**, *6*, 33.

[237] N. Cui, D. R. Friend, R. N. Fedorak, *Gut* **1994**, *35*, 1439.

[238] E. Cario, *Mucosal Immunol* **2012**, *5*, 2.

[239] S. Hua, E. Marks, J. J. Schneider, S. Keely, *Nanomedicine* **2015**, *11*, 1117.

[240] M. K. Chourasia, S. K. Jain, *Drug delivery* **2004**, *11*, 129.

[241] P. Artursson, T. Lindmark, S. S. Davis, L. Illum, *Pharm Res* **1994**, *11*, 1358.

[242] Y. Pan, Y. J. Li, H. Y. Zhao, J. M. Zheng, H. Xu, G. Wei, J. S. Hao, F. D. Cui, *Int J Pharm* **2002**, *249*, 139.

[243] E. L. Carvalho, A. Grenha, C. Remunan-Lopez, M. J. Alonso, B. Seijo, *Methods in enzymology* **2009**, *465*, 289.

[244] B. Sarmento, A. Ribeiro, F. Veiga, P. Sampaio, R. Neufeld, D. Ferreira, *Pharm Res* **2007**, *24*, 2198.

[245] F. M. Goycoolea, G. Lollo, C. Remunan-Lopez, F. Quaglia, M. J. Alonso, *Biomacromolecules* **2009**, *10*, 1736.

[246] M. R. Avadi, A. M. Sadeghi, N. Mohammadpour, S. Abedin, F. Atyabi, R. Dinarvand, M. Rafiee-Tehrani, *Nanomedicine* **2010**, *6*, 58.

[247] S. Al-Qadi, M. Alatorre-Meda, M. Martin-Pastor, P. Taboada, C. Remuñán-López, *Colloids and Surfaces B: Biointerfaces* **2016**, *141*, 223.

[248] L. Liu, C. Zhou, X. Xia, Y. Liu, *Int J Nanomedicine* **2016**, *11*, 761.

[249] Y.-H. Lin, K. Sonaje, K. M. Lin, J.-H. Juang, F.-L. Mi, H.-W. Yang, H.-W. Sung, *Journal of Controlled Release* **2008**, *132*, 141.

[250] P. Zhang, Y. Xu, X. Zhu, Y. Huang, *International Journal of Pharmaceutics* **2015**, *496*, 993.

[251] M. Lopes, N. Shrestha, A. Correia, M.-A. Shahbazi, B. Sarmento, J. Hirvonen, F. Veiga, R. Seiça, A. Ribeiro, H. A. Santos, *Journal of Controlled Release*.

[252] H. Laroui, G. Dalmasso, H. T. Nguyen, Y. Yan, S. V. Sitaraman, D. Merlin, *Gastroenterology* **2010**, *138*, 843.

[253] B. Xiao, H. Laroui, E. Viennois, S. Ayyadurai, M. A. Charania, Y. Zhang, Z. Zhang, M. T. Baker, B. Zhang, A. T. Gewirtz, D. Merlin, *Gastroenterology* **2014**, *146*, 1289.

[254] F. Bigucci, B. Luppi, T. Cerchiara, M. Sorrenti, G. Bettinetti, L. Rodriguez, V. Zecchi, *European Journal of Pharmaceutical Sciences* **2008**, *35*, 435.

[255] K. Mladenovska, R. S. Raicki, E. I. Janevik, T. Ristoski, M. J. Pavlova, Z. Kavrakovski, M. G. Dodov, K. Goracinova, *Int J Pharm* **2007**, *342*, 124.

[256] S. Wittaya-areekul, J. Kruenate, C. Prahsarn, *Int J Pharm* **2006**, *312*, 113.

[257] V. Araujo, A. Gamboa, N. Caro, L. Abugoch, M. Gotteland, F. Valenzuela, H. A. Merchant, A. W. Basit, C. Tapia, *J Pharm Sci* **2013**, *102*, 2748.

[258] J. das Neves, M. F. Bahia, *International Journal of Pharmaceutics* **2006**, *318*, 1.

[259] D. H. Owen, D. F. Katz, *Contraception* **1999**, *59*, 91.

[260] D. P. Wolf, J. E. Sokoloski, M. Litt, *Biochimica et biophysica acta* **1980**, *630*, 545.

[261] J. das Neves, R. Nunes, A. Machado, B. Sarmento, *Advanced Drug Delivery Reviews* **2015**, *92*, 53.

[262] S. L. McGill, H. D. C. Smyth, *Mol. Pharm.* **2010**, *7*, 2280.

[263] Ž. Vanić, N. Škalko-Basnet, *European Journal of Pharmaceutical Sciences* **2013**, *50*, 29.

[264] S. K. Lai, D. E. O'Hanlon, S. Harrold, S. T. Man, Y.-Y. Wang, R. Cone, J. Hanes, *Proceedings of the National Academy of Sciences* **2007**, *104*, 1482.

[265] L. Illum, N. F. Farraj, S. S. Davis, *Pharmaceutical Research* **1994**, *11*, 1186.

[266] A. M. Hillery, A. W. Lloyd, J. Swarbrick, "*Drug Delivery and Targeting: For Pharmacists and Pharmaceutical Scientists"*, Taylor & Francis, 2003.

[267] P. Tengamnuay, A. Sahamethapat, A. Sailasuta, A. K. Mitra, *International Journal of Pharmaceutics* **2000**, *197*, 53.

[268] C. Caramella, F. Ferrari, M. C. Bonferoni, S. Rossi, G. Sandri, *Journal of Drug Delivery Science and Technology* **2010**, *20*, 5.

[269] C. Prego, F. M. Goycoolea, "Nanostructures Overcoming the Nasal Barrier: Protein and Peptide Delivery Strategies.", in *Nanostructured Biomaterials for Overcoming Biological Barriers*, M.J. Alonso and N. Csaba, Eds., The Royal Society of Chemistry, 2012, p. 133.

[270] A. Vila, A. Sanchez, M. Tobio, P. Calvo, M. J. Alonso, *Journal of Controlled Release* **2002**, *78*, 15.

[271] B. Menchicchi, J. P. Fuenzalida, K. B. Bobbili, A. Hensel, M. J. Swamy, F. M. Goycoolea, *Biomacromolecules* **2014**, *15*, 3550.

[272] J. J. Homer, A. C. Dowley, L. Condon, P. El-Jassar, S. Sood, *Clinical Otolaryngology* **2000**, *25*, 558.